Cytokine Regulation and Development of Human Anti-malarial Immunity during *Plasmodium falciparum* Infections

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Doctoral Dissertation in Biological Sciences
The University of Edinburgh
1999

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Acknowledgements

Thanks to my advisors Professor Eleanor Riley, Dr. Chris Inchley and Dr. Alun Maddy for the guidance and support given during my dissertation research (A.K.A the hardest thing that I have done or ever will do).

Thanks in advance to Dr. David Dunne and Dr. Richard Carter for the arduous task of doing my viva.

Special thanks to everyone in Dr. David Walliker's group for providing the 3D7A line and for doing some of the mycoplasma testing.

Special thanks to Su Haley for teaching me the basics of schizont culture maintenance and more importantly, for filling all the tip boxes!

Thanks to Kevin Tetteh for his help in the PCR parasite detection assays.

Thanks to Kay Samuel and Martin Waterfall for running some of the FACs samples.

Thanks to all the people at Roslin for teaching me the basics of RT-PCR.

Special thanks to Steve Smith for helping me in finally getting the ICS to work!

Special thanks to all of my blood donors here in Edinburgh as well in Ghana (from your favourite vampire, I owe you all Mars bars if I pass).

Thanks so much to everyone in the Immunology department at the Noguchi Memorial Institute for Medical Research for collaborating on this project and more importantly, for making it so much fun to actually do lab work.

Special thanks to all of my friends (David Guilliano and Daphne Gerrits in particular) for helping me through the really rough periods of my work. I would have quit a long time ago if it weren't for all of you.

And a very special thanks to Simon for always being there with smiles even when I was going completely crazy.

This project was funded by the Wellcome Trust.

List of abbreviations

αβ	alpha beta
Ab	Antibody
Ag	Antigen
bp	base pairs
BSA	Bovine serum albumin
χ_2	Chi-squared test
CD	Clusters of differentiation (cell surface markers)
CD40L	CD40 ligand
cDNA	Complimentary DNA
CI	Confidence interval
CM	Culture medium
cpm	counts per minute
DC	Dendritic cells
df	degrees of freedom
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	dithiothreitol
ELAM-1	Endothelial leukocyte adhesion molecule 1
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
FACs	Fluorescent-activated cell sorting
FACs PBS	PBS supplemented with 0.1% NaN_3 and 0.1% BSA
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
γδ	gamma delta
g	Gram
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulfuric Acid
HRP	Avidin-labelled horse radish peroxidase
ICAM-1	Intercellular adhesion molecule 1
ICS	Intracellular cytokine staining
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IGIF	Interferon gamma inducing factor
IL-1	Interleukin one
IL-2	Interleukin two
IL-4	Interleukin four
IL-6	Interleukin six
IL-10	Interleukin ten
IL-12	Interleukin twelve
IL-15	Interleukin fifteen
IL-18	Interleukin eighteen
iNOs	Inducible nitric oxide synthase
IPTG	isopropyl-B-D-thiogalactopyranoside

List of abbreviations (continued):

iRBC	infected red blood cell
KO	Knock out
L	Liter
LLD	Lowest level of detection
LPS	Lipopolysaccharide
Mø	Macrophages
M	Molar
mg	Milligram
µg	Microgram
$MgCl_2$	Magnesium Chloride
ml	Milliliter
MHC	major histocompatability complex
µl	Microliter
mM	Millimolar
mRNA	messenger RNA
NaN_3	Sodium Azide
ND	Not done
ng	Nanogram
nm	Nanometer
nM	Nanomolar
NO	Nitric oxide
OD	Optical density
OPD	o-phenylenediamine
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PHA	Phytohaemagglutinin
PPD	Purified protein derivative of M. tuberculosis
RBC	Red blood cells
pg	Picogram
R	Receptor
R1	Resting cells
R2	Blasting cells
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SSC	Side scatter
SD	Standard deviation
SEB	Staphylococcal enterotoxin B
SEM	Standard error of the mean
SI	Stimulation index
TBE	Tris-borate-EDTA buffer
TcR	T cell receptor
TGF-β	Transforming growth factor beta
TNF-α	Tumour necrosis factor alpha
Tri	Tricolour
Tween 20	Polyoxyethylene sorbitan monolaurate
U	Unit
uRBC	Uninfected red blood cells
VCAM-1	Vascular cell adhesion molecule 1
X-gal	5-bromo-4-chloro-3-indolyl-B-D-thiogalactopyranoside

Abstract

Malaria is one of the most common infectious diseases in the world. In particular, *Plasmodium falciparum* malaria is responsible for more deaths than any other parasitic disease. Although there are preventative measures against malaria, it is hoped that a vaccine can soon be developed that will have long term benefits in malaria control. In order to design a safe and effective vaccine, there needs to be a more thorough understanding of the mechanisms involved in anti-malarial immunity and which immune responses are protective or pathogenic.

Individuals living in malaria-endemic areas develop clinical immunity. Although these individuals do not have complete sterilising immunity, they are able to control infection (as shown by the absence of clinical symptoms in the presence of parasites) and limit parasite replication. In contrast, unexposed, or naive, individuals inevitably develop clinical illness when infected with malaria. Elucidating the differences between these two patterns of response is the key to developing prevention strategies.

It is now widely accepted that clinical symptoms develop as a result of an excessive inflammatory response to malaria antigen. I hypothesise that inflammation is primarily due to a "Th-1-like" response and that the development of clinical immunity is associated with the downregulation of pro-inflammatory cytokines. I further hypothesise that immune responses of naïve and clinically immune individuals are mediated by different populations of mononuclear cells.

In order to test these hypotheses, I first developed specific and sensitive methods to measure cytokine production. I then compared proliferative responses and IFN-γ production of peripheral blood mononuclear cells (PBMCs) to *P. falciparum* schizont extract (PfSE) from malaria naive, malaria-exposed (but not clinically immune) and malaria-immune individuals. In order to determine how PfSE-induced IFN-γ production is regulated, I have also measured IL-12 p40, IL-12 p70 and IL-10 from PfSE-stimulated PBMCs and investigated the role of neutralising antibody to IL-12 in modulating IFN-γ production. Finally, a combination of cell surface staining and intracellular cytokine staining was used in order to determine the phenotypes of cells responding to PfSE.

Cells from all individuals proliferated vigorously in response to PfSE, but there were no significant differences between any of the groups. Cells from naïve individuals

produced moderate levels of IFN-γ which were mainly IL-12-dependent. Intracellular cytokine staining analysis indicated that IFN-γ was primarily produced by αβ+ T cells, although significant proportions of γδ+ T cells and NK cells also produced IFN-γ. IFN-γ levels were significantly higher in PBMC cultures of exposed individuals than in cell cultures from naïve individuals and were only partially IL-12-dependent. In contrast, minimal levels of IFN-γ were produced from PfSE-stimulated cells of immune individuals and were significantly lower than those found in either naïve or exposed populations. Neutralising antibodies to IL-12 reduced IFN-γ to background levels in cell cultures of immune donors. Although these data indicate that IL-12 does play a role in mediating IFN-γ responses, no significant differences were found in malaria-specific IL-12 p40 protein levels or mRNA transcripts between any of the groups. There was also no significant difference between the groups in IL-10 production, suggesting that this cytokine does not play a major role in modulating PfSE-stimulated IFN-γ production.

In cell cultures from all 3 groups, PfSE-stimulated cells were predominantly T cells although there were also activated NK cells and B lymphocytes. CD4+ αβ+ CD45RO+ cells were the predominant population of lymphoblasts in cell cultures of naïve individuals, although γδ+ cells also responded to PfSE. Although exposed individuals had the same predominant cell phenotype responding to PfSE, they also had significantly higher numbers of responding CD8+ cells than naïve individuals. The data from this study strongly supports the idea that the downregulation of inflammatory cytokines is a critical component of clinical immunity and that this may be dependent on the cell population which is activated.

These findings have important implications for the development of antimalarial vaccines; vaccination protocols should avoid the induction of strong pro-inflammatory immune responses in naïve individuals and should seek to target antigens recognised by the CD8+ population which may be associated with protective clinical immunity.

Chapter 1: Introduction

1.1 What is malaria?

1.1.1 *Plasmodium* life cycle

Malaria is a mosquito-borne infectious disease caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium falciparum* is the most common and most pathogenic type of the 4 species which infect humans. *Plasmodium* has 4 different growth stages (figure 1.1), 2 in mosquitoes (sexual and asexual) and 2 in humans (both asexual). After an infectious bite, sporozoites migrate to the liver where they undergo growth and reproduction. Infection is asymptomatic (3, 175, 210). After about 8 or 9 days, merozoites, the asexual erythrocyte stage which causes the clinical symptoms of malaria (208), are released and then infect red blood cells (RBCs). Fever occurs when schizont-infected erythrocytes burst and release merozoites which parasitise new RBCs. It is thought that toxins released at schizont burst, such as glycosylphosphatidylinositol (GPI)(117, 305), stimulate macrophages (Mø) to produce tumour necrosis factor alpha (TNF-α) which in turn mediates fever by upregulating the inflammatory response (188, 190, 192). However, the extent to which these toxins alone can mediate all the pathology of malaria has recently come into question (308).

1.1.2 Malaria: the problem

Malaria is the second most common infectious disease in the world. It is estimated that over 300 million cases and over 3 million malaria-related deaths occur every year, with the majority of cases occurring in sub-Saharan Africa (280). The majority of deaths occur in children, and malaria is a major cause of illness and death in pregnant women (100). In particular, severe anaemia and/or cerebral malaria are the common clinical manifestations of *P. falciparum* (218, 222, 229, 233, 280). Mortality is caused only by *P. falciparum*, because illnesses such as cerebral malaria are thought to result from sequestration (adherence of infected RBCs to endothelial cells), a phenomenon which does not occur with other *Plasmodium* infections (175).

Currently, the only forms of personal protection against malaria are prophylactic drugs, such as chloroquine and mefloquine, or prevention of direct contact with mosquitoes via use of insecticide-impregnated bed nets and mosquito repellents.

These forms of protection are highly problematic for several reasons. The emergence of drug-resistant strains of *Plasmodium* has reduced the efficacy of many chemoprophylactic regimens. Also, in underdeveloped countries where the highest prevalence of malaria occurs, individuals are unable to afford the high cost of medical treatment. Although the initiation of impregnated bed net programs has been successful in reducing the number of malaria-related deaths (64), there is a risk that individuals who are no longer constantly exposed may have lower natural immunity to malaria.

With all of these problems, combined with the fact that the incidence of malaria is increasing, it is hoped that a vaccine, or measures which can lessen malaria pathogenesis, can soon be developed. DNA vaccinations conferred partial protection to *Plasmodium yoelii* infection in mice (78), and pre-immunisation with an attenuated *Salmonella typhi* vaccine expressing the circumsporozoite protein of *P. falciparum* elicited strong proliferative and interferon-gamma (IFN-γ) responses *in vitro* (324). But vaccine development has been highly problematic because of antigenic polymorphism and antigenic variation and because specific antigens (Ag) are presented at different stages of the parasite life cycle (77, 187, 280). It has been suggested that development of a multi-stage vaccine would be preferred, because protective immune responses to different stages of the parasite life cycle would be induced (77). In order to develop such a vaccine, there needs to be a better understanding of parasite-host interaction and of what immune responses would be beneficial.

1.1.3 The epidemiology of malaria

In highly endemic areas of stable, perennial malaria, infants less than one year of age have few clinical episodes of malaria (6, 40, 108, 213, 347). This phenomenon may be attributable to factors such as the presence of fetal haemoglobin (256) or transplacental transfer of maternal immunoglobulin (44). However, this innate immunity wanes with age, and children under the age of 5 have the highest risk of clinical attacks, whereas all ages are affected in areas of low endemicity (low, unstable, seasonal transmission) (233). Those individuals who survive past childhood develop premunition, a state of non-sterilising immunity where there is an absence of clinical symptoms when parasites are present in the bloodstream (222, 233, 360). It is

generally thought that repeated and constant exposure is required for acquired immunity (222). However, Deloron *et al.* demonstrated that malarial immunity is long lasting even without constant exposure (73). It has also been hypothesised that individuals develop anti-toxic (lack of clinical symptoms) and anti-parasitic (inhibition of parasite replication) immunity separately (268). This point has been challenged by studies which demonstrated that very few children with asymptomatic infection had high parasite counts (155) and that febrile temperatures were always associated with high parasite density across all age groups (262).

The immune mechanisms that cause clinical illness and subsequent clinical immunity are not fully understood in human malaria infection. It is thought that clinical disease is a result of an excessive inflammatory response and that downregulation of this response leads to immunity (discussed in section 1.2.2). Understanding the factors which underlie premunition may be the first step in developing preventative measures against malaria.

1.2 The innate immune response and malaria infection

1.2.1 Innate versus adaptive immunity

There are two main parts to immunity: innate and adaptive. Innate, or natural, immunity works in an antigen non-specific manner by recognising common components of pathogens. Innate defence mechanisms are in place before infection, are rapidly activated and are not enhanced by prior exposure; that is to say, there is no immunological memory (18, 94, 95). The innate immune system provides a first line of defence against invading organisms.

Adaptive, or acquired, immunity is exquisitely Ag specific and may be long lasting. This line of defence is specifically induced by foreign Ag and relies upon clonal expansion of T and B lymphocytes (94, 95); as a result, adaptive immunity is not as immediate as innate immunity. Each successive exposure to such Ag enhances the immune response; i.e. immunological memory has developed, and results in more rapid secondary and subsequent responses.

The first interaction with an invading pathogen is via the innate immune system. Cells such as Mø (macrophages) and natural killer (NK) cells can directly lyse and/or phagocytose foreign pathogens (23). But more importantly, these cells produce

cytokines which affect the differentiation and proliferation of B and T lymphocytes (94, 95, 227, 333). Thus, the interaction between the innate immune system and a pathogen is involved in the initiation of subsequent adaptive immune responses.

1.2.2 Innate immunity and malaria

Malaria parasites activate IFN-γ production from NK cells which in turn stimulates Mø to produce TNF-α and to attack parasites (189). These responses are essential in limiting primary infection. In mouse models, IFN-γ inhibited parasite replication (98) and IFN-γ and TNF-α conferred protection against malaria via a nitric oxide (NO)-dependent pathway (151, 309, 320). Elevated serum levels of IFN-γ and TNF-α correlate with an absence of parasitemia (74) and rapid cure (182) respectively in human malaria.

However, overproduction of these cytokines may be what causes clinical illness. Mice infected with *Plasmodium berghei* (*P. berghei*) ANKA failed to develop cerebral malaria when IFN-γ and TNF-α were neutralised (293). In humans, plasma concentrations of these cytokines were higher in symptomatic than in asymptomatic infections (118, 193, 238, 356). Taken together, these observations suggest that there is a critical balance of the quantity, timing, location and context (i.e. cytokine milieu) of cytokine production, which determine if these responses are pathogenic or beneficial. Thus, the immune system is involved both in protective immunity and pathogenesis.

1.3 Components of *P. falciparum* antigen

1.3.1 Early *in vitro* studies with *P. falciparum* antigen

Individuals who live in endemic areas are continuously exposed to malaria. When cultured *in vitro*, their peripheral blood mononuclear cells (PBMCs) proliferate vigorously to malaria Ag (114, 281). More interestingly, it has also been shown that cells (studies have primarily used T cells) from individuals who have never been previously exposed to malaria, can also be induced to proliferate in response to plasmodial antigens (14, 20, 62, 63, 75, 97, 105, 112, 115, 119, 159, 290, 366, 370). The question that remains to be answered is: how is malaria recognised by these T cells? There are three different stimuli which are able to activate T lymphocytes: mitogens, superantigens and classical recall antigens.

1.3.2 Mitogens

Mitogens, which tend to be molecules derived from plant or bacterial cell walls, generate a significant lymphoproliferative response in T and B cells with the highest *in vitro* proliferation usually seen between 2 and 4 days. Mitogens bind directly to cell surface receptors without needing to be processed and presented on major histocompatability complexes (MHC). For example, phytohaemagglutinin (PHA), a plant lectin, binds directly to the CD3-T cell receptor (TcR) complex which is expressed by all T cells, whatever their Ag specificity, and transduces a proliferative signal (1). Thus, there is a rapid, non-specific, polyclonal activation of cells in response to mitogens.

Studies done in the late 70s-early 80s showed that *P. falciparum* extracts generated non-specific blast transformation *in vitro* of PBMCs from immune as well as non-immune donors (14, 105, 119, 366). Because unsensitised individuals were also able to respond, it was thought at the time that the primary mechanism was mitogenic. However, it was agreed that *P. falciparum* extracts also exhibited some antigenic activity.

1.3.3 Superantigens

Superantigens are interesting in that they have some characteristics of both mitogens and antigens. The most common superantigens, staphylococcal enterotoxins, are able to stimulate a significant proportion of T cells by cross-linking MHC with the variable portion of (V_β) of the T cell receptor beta chain (101). Superantigens are not processed and presented on MHC but do require MHC expression as they bind to MHC class II outside the peptide-binding site (101). Maximal *in vitro* proliferation is usually seen between 3 and 6 days. They are not able to stimulate as large a proportion of T cells as mitogens, because different superantigens stimulate cell with different V_β chains (1).

Behr *et al.* hypothesised that *P. falciparum* extracts are superantigenic rather than antigenic in nature, because the kinetics of the response they induce are most similar to those produced by enterotoxins (20). In addition, *P. falciparum* extracts induced a significant increase in gamma-delta T ($\gamma\delta$+) cells in cultures from malaria-unexposed donors (20). This is characteristic of the response to staphylococcal

enterotoxin B (SEB) but not to purified protein derivative of *Mycobacterium tuberculosis* (PPD), a classical recall Ag. Other studies also concluded that *P. falciparum* extracts had superantigenic components, because extracts stimulated a proportion of naïve (i.e. unprimed) T cells (97, 115). However as discussed in the next section (1.3.4), most studies demonstrate that *P. falciparum* extracts stimulate T cells in the same manner as a classical recall Ag.

1.3.4 Classical recall antigens

Antigens need to be processed and presented on MHC in order to be recognised by T cells. Maximal *in vitro* proliferation to Ag, such as PPD, is usually seen between 6 and 9 days. After primary stimulation, Ag-specific clones of T cells proliferate and a high frequency of memory T cells are produced that have exquisite specificity to a particular Ag. Thus, there is a much smaller proportion of T cells being activated in a recall antigenic response (1-5%) than in response to a superantigen (10-30%) or mitogen (100%).

There is a clear consensus that *P. falciparum* extracts are also antigenic in nature in the classical sense. In contrast to the results from Behr *et al.*'s study, others have demonstrated that proliferative kinetics are similar to other recall antigens (62, 63). Second, a number of studies also proved that responses to *P. falciparum* are not only MHC-*dependent* but are MHC-*restricted* (i.e. HLA-dependent restriction of T cell proliferation), a crucial component of Ag presentation (114, 159, 290).

If *P. falciparum* is truly antigenic in nature, why does the immune system respond so vigorously, if it has had no previous exposure? It is hypothesised that the responding cells are in fact memory cells. Memory cells are believed to be maintained at high frequency in peripheral blood through cross-reactive stimulation with other environmental Ag. Currier *et al.* and Bender *et al.* generated malaria-specific T cell clones from non-exposed individuals and found that these cells were able to proliferate in response to a number of common environmental organisms (24, 62, 63). This hypothesis is further supported by the fact that similar cross-reactive responses are found with yeast and other parasitic infections such as *Leishmania* (275), *Paracoccidioides brasiliensis* (239, 240) and *Trypanosoma cruzi* (266).

1.4 Cellular responses to malaria infection

1.4.1 Primary immune cells

1.4.1.1 Macrophages

Macrophages (Mø) are mononuclear phagocytes, which provide one of the first lines of defence in innate immunity (1). They also act as Ag presenting cells (APC) to CD4 cells, therefore stimulating the adaptive immune response (267). They are closely related to blood monocytes.

Mø produce a number of inflammatory cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α, and oxidative metabolites (NO discussed in section 1.5.3.4) which have been implicated in the pathogenesis of malaria (153, 190, 261), particularly TNF-α which is a mediator of fever paroxysms in acute *P. falciparum* infection (32, 55, 191, 194). Mø also produce interleukin-12 (IL-12), a cytokine which recruits NK cell for destruction of foreign pathogens, stimulates IFN-γ production by NK cells, and mediates T cell differentiation down a Th1 pathway (142, 144, 164, 301). IL-12 may play an important role in malaria infection because it can stimulate a Mø-mediated inflammatory response via IFN-γ and TNF-α (61).

Mø also regulate inflammatory responses by producing anti-inflammatory cytokines such as interleukin 10 (IL-10) and transforming growth factor beta (TGF-β) (66, 141). These cytokines decrease inflammatory cytokine production from PBMCs of *P. falciparum*-infected individuals (135) and in mice infected with *Plasmodium berghei* (252).

1.4.1.2 NK cells

NK cells are large granulocytic lymphocytes which represent about 15% of the total circulating lymphocytes in peripheral blood (1). Human stimulatory (CD16 and CD56) as well as inhibitory (KIR and CD94) NK cell Ag receptors mediate cytotoxicity when target cells fail to express MHC class I proteins on their cell surface (39, 123, 147, 209, 277, 278, 307)

One of their primary functions is to lyse foreign pathogens or infected cells (123, 278). NK cells in conjunction with Mø, provide the first line of defence in innate immunity. NK cells also facilitate the development of Th0 cells into Th1 cells via IFN-γ production (323). Most importantly, they are a major source of IFN-γ which is essential

in activating Mø to kill invading pathogens during the early stages of infection (143, 289, 302, 307). IFN-γ production by NK cells confers resistance in both *Toxoplasma gondii* and *Plasmodium chabaudi* infection (143, 236), but decreases resistance in mice infected with *Listeria* (331)

1.4.2 T lymphocytes

1.4.2.1 CD8+ T cells

CD8+ T cells, or cytotoxic T cells, lyse infected cells which express processed foreign Ag in association with MHC class I molecules. They also protect against parasitic infections such as *Toxoplasma gondii* through the production of IFN-γ (321). In malaria vaccination studies in mice, protection against exoerythrocytic stages of *P. yoelii* was absolutely dependent on IFN-γ from CD8+ cells (78). CD8+ cells are major IFN-γ producers and probably produce more IFN-γ than CD4+ on a cell to cell basis (363).

At first it was thought that CD8+ cells did not play a role in acute malaria infection, because infected red blood cells do not express MHC class I molecules. However, CD8+ cells may have some suppresser activity. While some studies showed that the removal of CD8+ T cells from PBMC cultures had no effect on lymphocyte proliferative responses to malaria Ag (136), others have clearly shown an increase in responsiveness in the absence of CD8+ cells (285). Although the exact mechanism of suppression is not known, CD8+ cells may be producing downregulatory cytokines such as TGF-β, a cytokine which is able to suppress proliferative responses (348).

1.4.2.2 Naïve and memory CD4+ αβ+ T cells

Greater than 90% of all circulating T cells bear T cell receptor (TcR) composed of α and β chains (239, 365). CD4+ cells, or helper T (Th) cells activate both Mø and B cell growth and differentiation (1). They may be further divided into subsets on the basis of cytokine secretion (see section 1.5.2). CD4+ αβ+ cells are required for maximal γδ+ cell activation (84, 199, 291, 319).

Numerous *in vitro* studies demonstrate that CD4+ αβ+ memory cells are the predominant cell population which proliferate in PBMCs from previously unexposed individuals exposed to different fungal, bacterial, and parasitic organisms, including

P. falciparum (62, 63, 75, 239, 240, 266, 290, 370). The majority of these cells express the RO isoform of CD45 (CD45RO+) (75, 239, 266) which is indicative of previously activated or memory cells (125, 159). These studies have led to the hypothesis that common pathogens or commensal or environmental organisms carry antigenic epitopes which cross-react with those of other organisms such that malaria-reactive T cell clones can be expanded by exposure to non-malarial antigens (63).

However some studies have also shown that cells bearing CD45RA, normally a naive cell marker, can also respond to malaria Ag. Goodier *et al.* suggested that the Ag preparation used in these experiments must contain some superantigenic elements since both memory and naive cells proliferate (115). In studies by Fern *et al.*, CD45RA+ is the only subset which expanded (97). These results suggest a large number of antigenic epitopes can be presented by MHC class II molecules, and this results in an unusually extensive T cell response. Alternatively, the CD45RA+ population identified by Fern *et al.* may have represented activated cells which had reverted to the naïve phenotype (125).

Depending on the type of infection and what stage the infection has reached, CD4+ $\alpha\beta$+ cells may stimulate an Ab-mediated or a cell-mediated inflammatory response. CD4+ cells, clonally expanded *in vitro* with *P. yoelii* and then transferred into reconstituted nude mice, were found to mediate a complete clearance of infection through IFN-γ (9). CD4+ $\alpha\beta$+ cells are also potent producers of interleukin-2 (IL-2) which in turn activates $\gamma\delta$+ cell-mediated production of IFN-γ. However, if CD4+ $\alpha\beta$+ cells produce too much IFN-γ, pathology may result from excessive production of other inflammatory cytokines such as TNF-α (293). However, CD4+ cells can also produce anti-inflammatory cytokines such as IL-4 which downregulate or inhibit the downstream effects of inflammatory cytokines or which help mediate Ab production. Murine *Plasmodium chabaudi chabaudi AS* infection (197) and human *P. falciparum* infection is resolved by antibody production (57)

1.4.2.3 $\gamma\delta$+ T cells

T cells also recognise Ag through a TcR composed of γ and δ chains, but only 6-8% of lymphocytes in human peripheral blood express $\gamma\delta$ receptors. (365). A small proportion of $\gamma\delta$ cells are CD4+ or CD8+, but most are CD4-/CD8-. In adult humans,

two-thirds of these cells express the Vγ9 and Vδ2 chains (112, 365). However, Vγ9Vδ2+ cells are not the major subset of γδ cells in cord blood or in intestinal epithelia (69, 112), suggesting that these cells may be expanded by constant antigenic exposure. Although the Vγ9 Vδ2 is the largest subset in healthy individuals, and is usually the one which is expanded during malaria infection (20), in one report the Vγ9 Vδ1 subset was shown to be preferentially expanded during *P. falciparum* infection (51).

γδ+ cells differ from αβ+ T cells in the way they recognise Ag, since γδ+ cells are able to recognise nonpeptidic substances. Though some αβ+ cells are able to do the same (165), they are MHC restricted, whereas γδ+ cells are not (35, 69, 106, 107). γδ+ cells can recognise protein Ag in one of three ways. First, they can recognise processed peptide in a classical MHC-*restricted* fashion; Haregewoin *et al.* found that γδ+ clones are unable to respond to HLA mismatched APC (127). Second, they recognise Ag in a MHC-*dependent* manner; that is, MHC must be present but does not have to be HLA-matched. For example, Schild *et al.* showed that a γδ+ clone specific for one MHC was able to cross-react with other MHC molecules (304). Finally, γδ+ cells recognise extracellular peptides or non-peptide Ag which bind directly to the TcR without the need for MHC presentation (35, 294). Ag recognition involves the Ag receptor, but γδ+ cells recognise conformational epitopes in an 'immunoglobulin-type' manner (294, 304).

γδ+ cells may play a role in immunosurveillance, because they are able to detect and destroy infected cells and because of their high prevalence in epithelial locations (127, 294). Elloso *et al.* showed that γδ+ cells inhibit *P. falciparum* merozoites from infecting erythrocytes *in vitro*, thereby inhibiting active malaria infection (85). In αβ+ TcR-deficient mice, γδ+ cells are able to lyse *T. gondii* -infected Mø (163). More importantly γδ+ cells participate in the inflammatory response through the production of pro-inflammatory cytokines such as IFN-γ (17, 54, 106, 107, 116). γδ+ cells produced high levels of IFN-γ, which mediated the clearance of murine *T. gondii* infection (163). *In vitro* studies showed that human γδ+ cells are stimulated to produce IFN-γ against malaria Ag (116, 264) and concluded that γδ+ may have an immunopathologic role.

There is a significant increase in the proportion of γδ+ cells in peripheral blood during acute malaria infection (137, 242, 260, 291, 295), although in two studies, no

increase in γδ+ cell proportions were found during infection in individuals who have lived all their lives in malaria endemic areas (145, 260). Thus γδ+ cell expansion may be a feature of acute malaria in non-immune or semi-immune individuals but the response may be down-regulated in clinically immune individuals. It has also been found that γδ+ cells are preferentially expanded *in vitro* when stimulated with *P. falciparum* Ag (20, 115, 198). These results seem to contradict the evidence that CD4+ αβ+ cells were preferentially expanded in response to malaria Ag (62, 63, 75, 290, 370). One reason for this discrepancy may be the source of malaria Ag used in the assays. Waterfall *et al.* demonstrated that CD4+ αβ+ cells are preferentially expanded when cells are cultured with a freeze-thaw schizont extract, while γδ+ cells are expanded in the presence of either live schizonts or schizont extract plus IL-2 (354). Elloso *et al.* also demonstrated that γδ+ cells only responded in the presence of CD4+ αβ+ cells (84). These results imply that CD4+ αβ+ cells may be the crucial cells stimulated, because γδ+ cell expansion requires CD4+ αβ+ cells for endogenous IL-2.

1.4.3 B cells

B cells represent only 15% of the total circulating lymphocytes in peripheral blood, but are much more abundant in lymph node (25%) and spleen (45%) (1). B cells may contribute to humoral immunity against malaria by generating Ag-specific antibodies but may also act as antigen-presenting cells for CD4+ cells. Vice versa, CD4+ cells produce T helper 2 (Th-2)-like cytokines (i.e. interluekin-4 (IL-4)) and T helper 1 (Th-1)-like cytokines (i.e. IFN-γ) which stimulate B cell production of neutralising and complement fixing antibodies respectively (2). The study of immunoglobulin production in malaria infection was pioneered by Cohen and McGregor in the early 1960s when they were able to demonstrate complete sterilising immunity to *P. falciparum* infection in African individuals who had received serum from immune individuals (57). Immunity by passive transfer of IgG in immune serum to non-immune adults has also been confirmed by Druilhe *et al.* (79). Antibodies are thought to confer protection against malaria by neutralising parasites (i.e. inhibiting merozoite RBC invasion) through Ab-dependent cellular cytotoxicity (221) or through interactions with malaria 'toxins'. Although early acute infection is controlled primarily by pro-inflammatory cytokines, complete resolution of parasitemia requires antibody help in

mice infected with *P. c. chabaudi* (196, 330). In humans studies, higher levels of IgG and IgM are found in individuals living in malaria-endemic areas (222), though these antibodies may not all be parasite specific. Riley *et al.* also found that Ab responses to merozoite surface protein-1 (MSP-1) increase with age (281). Finally, Luty *et al.* demonstrated that elevated levels of specific IgG1 and IgG3 were associated with lower or absent parasitemia in Gabonese schoolchildren (211). Taken together, these results suggest a protective role for B cells in malarial infection.

1.5 Cytokines mediating the immune response to malaria

1.5.1 Introduction

Cytokines are hormone-like proteins which provide a communication network within the immune system and mediate almost every aspect of innate and adaptive immunity. Monokines are the subset of cytokines which are mainly produced by mononuclear phagocytes. They control the effector functions of these cells and act as co-stimulators for lymphocyte activation. Lymphokines are the subset of cytokines which are produced by activated T lymphocytes; they regulate activation, growth, and differentiation of lymphocyte subsets. However, lymphokines also activate and regulate the inflammatory response.

The cytokine network is a complex series of interactions whose picture still remains incomplete. There are numerous cytokines which are produced when cells are stimulated with *P. falciparum* (116, 128, 349), many of which have overlapping and redundant functions. The question of whether infection is cleared or disease is exacerbated depends on the balance of the overall cytokine milieu.

1.5.2 The Th1 vs. Th2 paradigm

Studies in mice have demonstrated that CD4 T cells can be divided into Th1 and Th2 cell subsets based on the cytokines they produce and their primary functions. Th1 cells produce IFN-γ and IL-2 and mediate cellular immunity, whereas Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (IL-10 only in mice) and promote growth and differentiation of B lymphocytes (2, 91, 258, 288, 318). It is likely that this dichotomy also occurs in CD8 cells, as has been recently suggested in *Schistosoma mansoni* infection (91). It was originally thought that Th1 responses were mainly protective against bacterial,

protozoal and fungal pathogens while Th2 responses were mainly protective against helminth infections, although more recent studies have shown that both Th1 and Th2 cytokines mediate immune responses in many different types of infections (8, 301, 362). Recent studies have also shown that the Th1 vs. Th2 paradigm holds true for human CD4 cells, although the range of cytokines is less polarised (71, 86). For example, in humans IL-6 and IL-10 are produced by both Th1 and Th2 cells (318, 324).

Activated T cells are believed to express an early undifferentiated phenotype (Th0), which produces both types of cytokines (i.e. IFN-γ and IL-4) before further differentiation into Th1 or Th2 cells (86, 253). However, differentiated phenotypes do not always remain stable. For example, Sornasse *et al.* and Szabo *et al.* demonstrated that Th2 cells were able to revert to Th0 or Th1 cells under the influence of IL-12 (318) or IFN-γ (323). Although categorising Th cells based on the cytokines they produce is extremely helpful in describing immune responses, recent studies indicate that immune responses are not as polarised as has been thought (8). Rather it is the balance between cytokines that determine which effector mechanisms will predominate.

There is some evidence that Th0 cells include a distinct population of differentiated cells which do not function as precursors for Th1 and Th2 cells and which produce both Th1 and Th2 cytokines. A number of studies have identified cells producing a combination of IL-2, IL-4, IL-5, and IFN-γ (86, 234, 253, 265).

Finally, cells that produce only TGF-β have been designated Th3 cells. Th3 cells are downregulatory cells which immunosuppress Th1 and Th2 cellular responses via production of TGF-β (171, 220, 338, 361).

The nature of the Ag and the cytokine environment determine the pathway of differentiation of a naive Th cell. Sartono *et al.* found that cells from lymphatic filariasis patients produced Th1 cytokines when stimulated with PPD and Th2 cytokines when stimulated with worm Ag *in vitro* (299). Type 1 cytokines, IL-12 and IFN-γ, and type 2 cytokines, predominantly IL-4, will drive naive Th cells to differentiate into Th1 and Th2 cells respectively, while reciprocally downregulating the other class of cytokines (323).

The following sections describe some of the cytokines which have been identified as important in the context of malaria infection, focusing particularly on their roles in inflammatory responses.

1.5.3 Inflammatory cytokines

1.5.3.1 Cytokines produced by macrophages

IL-1

IL-1 (IL-1α and IL-1β) are produced by Mø to act both upon lymphocytes and upon Mø themselves. IL-1 stimulates a Mø-mediated inflammatory response, and like TNF-α, is also an endogenous pyrogen which mediates fever production by increasing prostaglandin synthesis by hypothalamic cells (1, 191). IL-1 acts as a co-stimulator for NO production by Mø (287) and for IFN-γ production by NK cells and T lymphocytes (66, 121, 142).

IL-1β and IL-12 mediate resistance via an IFN-γ pathway to *T. gondii in vivo* in mice with severe combined immunodeficiency (SCID) (142). A similar pathway could operate in malaria. However, elevated serum concentrations of IL-1 receptor antagonist, a soluble factor which competes with IL-1 for IL-1 receptors, were found in Gambian children with cerebral malaria and acute *P. falciparum* malaria but not asymptomatic infection, indicating that an overproduction of this inflammatory cytokine may be detrimental to the host (153, 154). Thus the balance of IL-1 production may be critical with low levels being beneficial and promoting parasite clearance and high levels promoting disease.

IL-6

IL-6 is also a Mø-derived pyrogen that helps stimulate the inflammatory response, although it is also considered to be a Th2 cytokine in mice and has an important role in B cell differentiation (1). IL-6, in conjunction with TNF-α, is a crucial cytokine in fever induction and innate protection against malaria parasites (182, 191). An early increase of serum IL-6 was demonstrated in protected vaccinees immunised with *P. falciparum* sporozoites (128). However, higher levels of IL-6 were found in individuals with complicated *P. falciparum* malaria and cerebral malaria compared to asymptomatic and aparasitemic individuals (15, 153, 182, 238).

TNF-α

TNF-α is an inflammatory cytokine which is produced primarily by Mø following contact with microbial pathogens (190) but also by NK cells and T lymphocytes. TNF-α

activates Mø and cytotoxic T cells for cellular killing, stimulates IL-1 and IL-6 production from Mø and acts as a co-stimulator of IFN-γ production by NK cells (311, 335). TNF-α contributes to both protection and pathology in different diseases.

TNF-α-mediated IFN-γ production from NK cells has been found to be necessary in clearing infections of *Listeria monocytogenes, T. gondii, and P. c . chabaudi* AS (121, 311, 320, 335). Although low levels early on in infection correlate with resistance, excessive levels of TNF-α late in *P. c. chabaudi* infection correlate with susceptibility in B6 mice, (152).

In humans, TNF-α is the primary endogenous pyrogen which causes fever in *P. falciparum* infection (55, 328). Kwiatkowski hypothesised that fever induced by TNF-α may be beneficial to the host, because elevated temperatures kill off the mature erythrocytic stages of the parasite (188, 190). It has also been demonstrated that monocyte-mediated Ab-dependent killing of *P. falciparum* is dependent on TNF-α (37). Kremsner *et al.* were able to correlate elevated levels of TNF-α with a more rapid clinical and parasitologic cure in patients with mild *P. falciparum* malaria (182). However, excessive levels of TNF-α can play a pivotal role in the pathogenesis of infectious disease. Groenveld *et al.* found high levels of numerous inflammatory cytokines, including TNF-α, in patients with severe sepsis (120). Elevated levels of TNF-α and TNF-α receptor 1 were found in individuals with acute and severe *P. falciparum* infection compared with individuals with asymptomatic infection (118, 154, 155, 193, 261).

TNF-α may be the major pathogenic cytokine in severe malaria. TNF-α suppresses bone marrow stem cell division and has been implicated in severe malarial anaemia (186). TNF-α increases lactate and decreases glucose levels which, if in excess, can lead to hypoglycaemia (56). Finally, TNF-α upregulates the adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) (28, 225), endothelial leukocyte adhesion molecule 1 (ELAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (248). It has been hypothesised that cerebral malaria is caused by the adherence of parasitised RBCs to these adhesion molecules in brain capillaries, causing reduced blood flow and hypoxia (248).

IL-12

IL-12, originally called NK cell stimulatory factor, is composed of 2 subunits, p35 and p40 which give rise to the biologically active p70 heterodimer. The p35 subunit is constitutively expressed by a number of cell types, while p40 is only produced by cells producing bioactive IL-12 (43, 124, 212, 243). It is produced primarily by monocytes, dendritic cells and Mø (124).

IL-4, IL-10, and TGF-β downregulate the production of IL-12 (219, 237, 301), and IFN-γ, which is itself upregulated by IL-12, upregulates IL-12 (113, 315, 333), although IL-12 can be produced independently of IFN-γ (130). IL-12 stimulates the production of all IgG isotypes (195, 230, 237), acts as a co-stimulator for T lymphocyte proliferation (132) and most importantly, activates the inflammatory response by enhancing NK cell maturation, cellular cytotoxicity and IFN-γ production (25, 26, 130, 237, 301). IL-12 also potentiates innate inflammatory response by driving Th0 cells to differentiate into Th1 cells, which also produce IFN-γ (13, 72, 306, 340).

IL-12 is produced in response to bacterial and parasitic infections, including *Leishmania braziliensis, M. tuberculosis* and *P. c. chabaudi* AS (316, 320, 326). Flynn *et al.* demonstrated that IL-12 is necessary for clearance of *M. tuberculosis* infection in BALB/c mice (102). Resistance to *Leishmania major* infection is correlated with the ability to produce IL-12 (21, 122), and IL-12 enhances the efficacy of a pertussis whole-cell vaccine (217). However, Taha *et al.* demonstrated that IL-12 levels increased in patients with active clinical tuberculosis, possibly indicating a detrimental effect of IL-12 (326).

Administration of recombinant IL-12 before sporozoite challenge conferred complete sterile protection against *P. cynomolgi* infection in monkeys (138). In resistant mice, the appearance of Th1 cells and high levels of IFN-γ, mediated by IL-12, predicts survival of the host and a relatively mild infection of *P. c. chabaudi* AS while the exact opposite occurs if Th2 cells are preferentially expanded (309, 320). Other studies have shown that IL-12 mediates killing not only of infected erythrocytes but also of infected hepatocytes (61). However, excessive levels of IL-12 may be pathogenic. Yoshimoto *et al.* demonstrated that IL-12 mediated clearance of blood stage *P. berghei* XAT (an attenuated strain) (369), but that in lethal *P. berghei* infection, IL-12 was involved in liver

damage and death (368). No data is available on whether the same is true for human malaria infection.

1.5.3.2 Cytokines produced by lymphocytes

IL-2

IL-2 is mainly produced by the Th1 subset of CD4+ and CD8+ cells. IL-2 is a potent stimulator of NK cells (334), but mainly induces the proliferation, differentiation, and activation of T lymphocytes (i.e. CD4+, CD8+ and γδ+ cells). It binds to the IL-2 receptor complex on T cells which causes the cells to move from G1 to S phase (332). γδ+ T cells proliferate in response to IL-2, or other cytokines that signal through the IL-2 receptor, when CD4+ αβ+ cells are stimulated with *P. falciparum* Ag *in vitro* (84). However, the role of IL-2 in pathogenesis is not clear. Mice susceptible to cerebral malaria had lower mRNA levels of IL-2 as compared to resistant mice (67), and IL-2 knockout mice had exacerbated *P. c. chabaudi* and *P. yoelii* infection as compared to controls (341). The absence of, or low levels of, IL-2 have been correlated with severe *P. falciparum* infection *in vivo* and human immunodeficiency virus HIV infection *in vitro* (135) (357). However, it should be noted that measuring IL-2 in plasma or cell culture supernatants is not a good indicator of how much IL-2 is being produced, because it rapidly binds to IL-2 receptors (IL-2R) and is removed from the fluid phase (1). Thus, soluble IL-2R is a better marker of IL-2 production.

IFN-γ

IFN-γ, or macrophage activating factor, is a non-covalently linked homodimer which has a number of structural variants (255). IFN-γ is produced by NK cells and T cells (both αβ+ and γδ+ cells (1), and its primary roles are Mø activation and differentiation of Th0 to Th1 cells (113). Although IFN-γ can be produced via an IL-12-independent pathway (300). IL-12 and IFN-γ stimulate and regulate each other through a positive feedback loop to both NK cells and Th1 cells, leading an amplification of the inflammatory response (315).

Immunity to various diseases, particularly those caused by intracellular pathogens, has been shown to be dependent on IFN-γ. NK cell production of IFN-γ is essential in clearing *L. major, L. monocytogenes, and T. gondii* infections (301, 311,

335), and resistance against *P. c. chabaudi* AS requires IFN-γ (309, 320). *P. yoelii* specific T cell clones which produce IFN-γ are protective when adoptively transferred into nude mice (9). Studies in monkeys have shown that IFN-γ is able to confer protection by inhibiting malaria parasite development in hepatocytes (216).

However, overproduction of IFN-γ may exacerbate the pathology of diseases such as malaria and tuberculosis in humans (75, 326). IFN-γ overproduction, secondary to lipopolysaccharide (LPS)-induced IL-12, is responsible for endotoxic-induced shock (334). In one mouse malaria model, IFN-γ receptor-deficient mice did not get cerebral malaria (293) and IFN-γ mRNA levels were higher in susceptible mice compared to controls (67). IFN-γ induced death when it was administered in the late stage of *Plasmodium vinckei* malaria (181). As with other inflammatory cytokines, pathology versus protection is dependent upon how much, where and when IFN-γ is produced.

1.5.3.3 Roles for IL-15 and IL-18

IL-15

IL-15 is an inflammatory cytokine which is mainly produced by Mø, although it is also produced by other types of cells. It has many functions which overlap those of IL-2, because IL-15 shares some of the same receptor components. It may act when IL-2 is downregulated (325). IL-15, alone and in conjunction with IL-12, controls the survival and apoptosis of NK cells (45, 289). It stimulates the production of IFN-γ by NK cells and αβ+ and γδ+ T lymphocytes (106, 143, 160). IL-15 is often associated with Th1-mediated clearance of intracellular pathogens such as *T. gondii*, because of its ability to induce IFN-γ (143). No studies have been done on the role of IL-15 in malaria infection.

IL-18

Recently, IL-18, formerly known as IFN-γ-inducing factor (IGIF), was discovered (250). It is produced mainly by Mø in an inactive form which is converted to the active form by caspase 1 (249). IL-18 induces NK cell production of IFN-γ and acts as a co-stimulator in the activation of Th1 cells (232). IL-12 and IL-18 can activate NK cells independently of each other which may explain IL-12-independent IFN-γ production from NK cells (179). However, the amount of IFN-γ produced is much higher when both

IL-12 and IL-18 are present (179, 232). IL-18 has been associated with IFN-γ-mediated clearance of the intracellular parasite, *Trypanosoma cruzi* (231). No data is available on the role of IL-18 in malaria infections.

1.5.3.4 Nitric oxide in the inflammatory response

NO is one of the primary reactive nitrogen intermediates which is produced by Mø during an inflammatory response. NO production is stimulated by TNF-α and IFN-γ induction of nitric oxide synthase (309, 320) and is downregulated by cytokines such as IL-4, IL-10 and TGF-β (34, 76, 151, 343). NO takes part in the inflammatory response to a number of bacterial, viral and parasitic infections by cytostasis (157, 158), but it is not clear how vital it is for parasitic killing. NO was required in clearing *P. c. chabaudi* AS (309, 320), *P. vinckei* (181) *Trypanosoma cruzi* (110) and *Klebsiella pneumoniae* (337) infections in mice, but in human malaria infection, it has been suggested that overproduction of NO may cause cerebral malaria. NO levels were higher in patients with severe *P. falciparum* malaria than in patients with asymptomatic infection (247). However, higher levels of NO were found in malaria patients who healed their infections versus coma patients (59), and were correlated with rapid cure (182). In a study done in Tanzania, NO levels were inversely correlated with severity of disease in individuals acutely infected with *P. falciparum* malaria (11).

1.5.4 Downregulatory cytokines

1.5.4.1 IL-4

IL-4 induces other CD4 cells to differentiate into Th2 cells by downregulating IL-12 receptor (IL-12R) expression thereby rendering cells non-responsive to IL-12 (113, 254). IL-4 is a B lymphocyte growth factor and has effects which are antagonistic to both IFN–γ and IL-2 (235). Although some studies show that IL-4 enhances IFN-γ synthesis early in the response to PHA (245) and that it synergises with IL-12 to induce a cytotoxic T lymphocyte response (132), suggesting that IL-4 does not always play an anti-inflammatory role.

IL-4 and other Th2 cytokines may play a protective role in parasitic infections by downregulating the inflammatory response and generating an Ab-mediated response. However, the results of different studies are mixed. Studies comparing control mice with

IL-4 knockout mice infected with *P. c. chabaudi* demonstrated either that there was no difference in parasitemia (341) or that infection was more severe in IL-4 knockout mice but that they were still able resolve infection (346). These studies indicate that immunity may not depend solely on IL-4 and that other anti-inflammatory cytokines may be involved, although IL-4 may be needed to set the cytokine environment.

IL-4 receptor levels were higher in Gambian children who had symptomatic *P. falciparum* infections than in children with asymptomatic infections, indicating that IL-4 may play a pathogenic role in malaria infection or that there is little immune activation of any sort in asymptomatic children (154). However, clinically immune individuals had higher ratios of IL-4/IFN-γ producing cells in response to a leucoagglutinin than naïve individuals (82), and IL-4 was not detected in cultures of PBMCs from naive individuals stimulated with *P. falciparum* Ag (75).

1.5.4.2 IL-10

IL-10, produced by Th2 cells (in mice) and Mø, has primarily anti-inflammatory functions(134, 135, 166, 263, 356). Although in certain circumstances it can synergise with pro-inflammatory cytokines to activate Th1 CD4+ cells (228) and to induce IFN-γ production from NK cells (46, 312). IL-10 is characterised by its ability to downregulate IL-1, IL-12, TNF-α and IFN-γ production, thereby preventing the immunopathogenic consequences of excessive inflammation (66, 144, 261).

In murine malaria infections, the role of IL-10 is unclear. IL-10 KO mice infected with *P. c. chabaudi AS* showed higher levels of parasitemia and increased mortality compared with wildtype mice (206), but similar mice infected with *P. chabaudi adami* demonstrated no increased susceptibility (341). Exogenous IL-10 reduced cerebral malaria in mice susceptible to *P. berghei* infection (180), but mice with lethal *P. yoelii* infections had high levels of circulating IL-10 (176).

In human malaria, high levels of circulating IL-10 correlate with disease severity (11, 15, 154, 263, 356), but low levels of IL-10 were associated with severe malarial anaemia (186).

1.5.4.3 TGF-β

The role of TGF-β in infectious diseases is complicated in that at low concentrations it is pro-inflammatory, and at high concentrations it is anti-inflammatory. Although, TGF-β is mainly produced by Mø (12, 348), it is also produced by almost any cell that is put into culture (1, 52). Th3, or regulatory T, cells which have specific immunosuppressive functions produce TGF-β (171, 220, 338, 361). TGF-β is secreted in a latent form which must be activated by proteases and is neutralised by binding proteins released by both activated and inactivated Mø (12). TGF-β immunomodulates the differentiation of B and T cells; it can stimulate or inhibit growth and differentiation of the same cells depending on its concentration (251, 252, 348). TGF-β also helps mediate the shift towards the Th2 phenotype both directly and by modulating IL-10 (214, 215). TGF-β has been shown to down-regulate the activation of cytotoxic T cells (132), NK cells (22, 141), and Mø production of inflammatory cytokines (33, 141) and NO (76, 343). However, TGF-β can also augment NK cell activity when it is given orally but not intravenously (149), indicating its dual role in immunomodulation.

TGF-β is often associated with pathology in parasitic infections because it down-regulates inflammatory cytokines. The inhibition of IL-12-induced IFN-γ by TGF-β abrogated resistance to *T. gondii* infection (141). TGF-β made naturally resistant mice susceptible to infection with *Leishmania major* (16) and *T. cruzi* (314). However, Omer *et al.* found that treatment of lethal *P. berghei* with TGF-β prolonged survival time (252). Additionally, Wenisch *et al.* measured TGF-β levels in patients with acute *P. falciparum* malaria and found that serum levels were lower than in uninfected controls (355).

1.6 Conclusions: the immune response during malaria infection

Adults living in highly endemic areas do not become clinically ill with malaria (i.e. they are classified as clinically immune), whereas individuals who have been infected but who are not clinically immune ('semi-immune' or exposed) and individuals who have never been infected (naïve) will become sick if infected with malaria. Hence, there must be a difference in the immune response to malaria between these groups of individuals.

During *P. falciparum* infection, it was originally thought that there is an initial Mø-dependent inflammatory response. When Mø come into contact with malaria Ag,

TNF-α is released and mediates fever. Other inflammatory cytokines, such as IL-1, IL-6, and IL-12, are also produced to upregulate the inflammation (figure 1.2). Although low levels of inflammatory cytokines help mediate parasite clearance in the early stages of infection, excessive levels have been associated with malaria pathogenesis (i.e. cerebral malaria, hepatosplenomegaly, anaemia, hypoglycaemia).

However, this scenario, or a totally innate response to malaria infection, does not explain why young infants do not become clinically ill during primary infection, as would be expected if the initial innate immune response alone caused excessive pathology. Recent studies have demonstrated that PBMCs from naïve individuals do not produce as much TNF-α and IL-12 in response to malaria Ag as in response to LPS, a bacterial pyrogen (308). TNF-α alone was not sufficient to neutralise the infectivity of gametocytes in *P. vivax* infections, demonstrating that inactivation was also dependent on other factors (358). Indeed levels of TNF-α induced directly by malaria parasites are very low and may be insufficient to cause a major febrile illness. Thus, there must be other factors in addition to parasite toxins which causes pathology in young children living in endemic areas and in naïve individuals.

PBMCs from naïve (malaria-unexposed) adults proliferate in response to malaria Ag, suggesting that T cells may play an important role in the early response to malaria. Carter *et al.* showed that activation of monocytes against *P. vivax* infection was dependent upon IL-2 and granulocyte-macrophage-colony stimulating factor (GM-CSF), cytokines produced predominantly by T cells (49). CD4+ αβ+ cells may respond quickly to malaria infection in adults who have a large population of primed cross-reactive T cells or in children who have previously been exposed to malaria and thus have a primed T cell response. These cells then proliferate and produce IFN-γ and IL-2. γδ+ cells bind malaria Ag, and in the presence of IL-12, secrete IFN-γ. IFN-γ from both CD4+ αβ+ and γδ+ cells stimulate macrophages to produce inflammatory cytokines as mentioned above (figure 1.3). Thus, it has been hypothesised that, in naive individuals, the inflammatory response may be upregulated and continually exacerbated by cross reactive memory T cells producing Th1-like cytokines.

Because, immune individuals are constantly exposed to malaria, they are able to mount an Ag-specific immune response to the invading pathogen. In naives, semi-immunes and immunes, there is probably an initial inflammatory response, with Mø

and NK cells being activated. But in immune individuals, this inflammatory response is probably quickly downregulated and memory T cells activate a Th2-like, Ab-mediated response (figure 1.4).

If there truly are different responses in terms of T cell subsets and cytokine responses between immune individuals and naïve individuals, these factors might explain the differences between the immune responses which cause illness and those which confer protection. In summary, a plausible hypothesis for the development of clinical immunity to malaria is that: (i) proinflammatory responses, mediated by primed populations of $\alpha\beta$+ and $\gamma\delta$+ T cells, cause a febrile illness in non-immunes, but that (ii) these inflammatory responses are downregulated in clinically immune individuals and (iii) replaced with anti-parasitic mechanisms which may include the production of highly effective anti-malarial antibodies. If these hypotheses prove correct, then there are important implications both for the treatment of clinical malaria and for the development of malaria vaccines

1.7 Aims and outline of the thesis

This scenario for the development of clinical immunity to malaria is, for the moment, entirely hypothetical. I aim to determine whether there are differences in cellular responses between naïve, semi-immune and immune individuals at an *in vitro* level. I hope that if my hypothesis prove correct, I can make some broader conclusions about what might happen *in vivo*. Specifically, the aims of the project are to test the following hypotheses:

a) that the development of clinical immunity is associated with the downregulation of pro-inflammatory cytokines.
b) that immune responses of each group (naïve, semi-immune and clinically immune) are controlled by different mononuclear cells.

The specific objectives of this project are:

a) to develop sensitive and specific methods for detection of pro-inflammatory human cytokines, especially for IL-12 which has been difficult to quantify in the past.

b) to investigate the cellular source of IFN-γ production from malaria-activated human cells and to determine how IFN-γ production is regulated by other cytokines, especially IL-12.

c) To compare lymphoproliferative and cytokine responses to malaria antigens in individuals with differing levels of anti-malarial immunity, in order to obtain preliminary evidence to support or refute the hypothesis defined above.

Figure 1.1: Life cycle of *Plasmodium falciparum* (taken from source (3)).

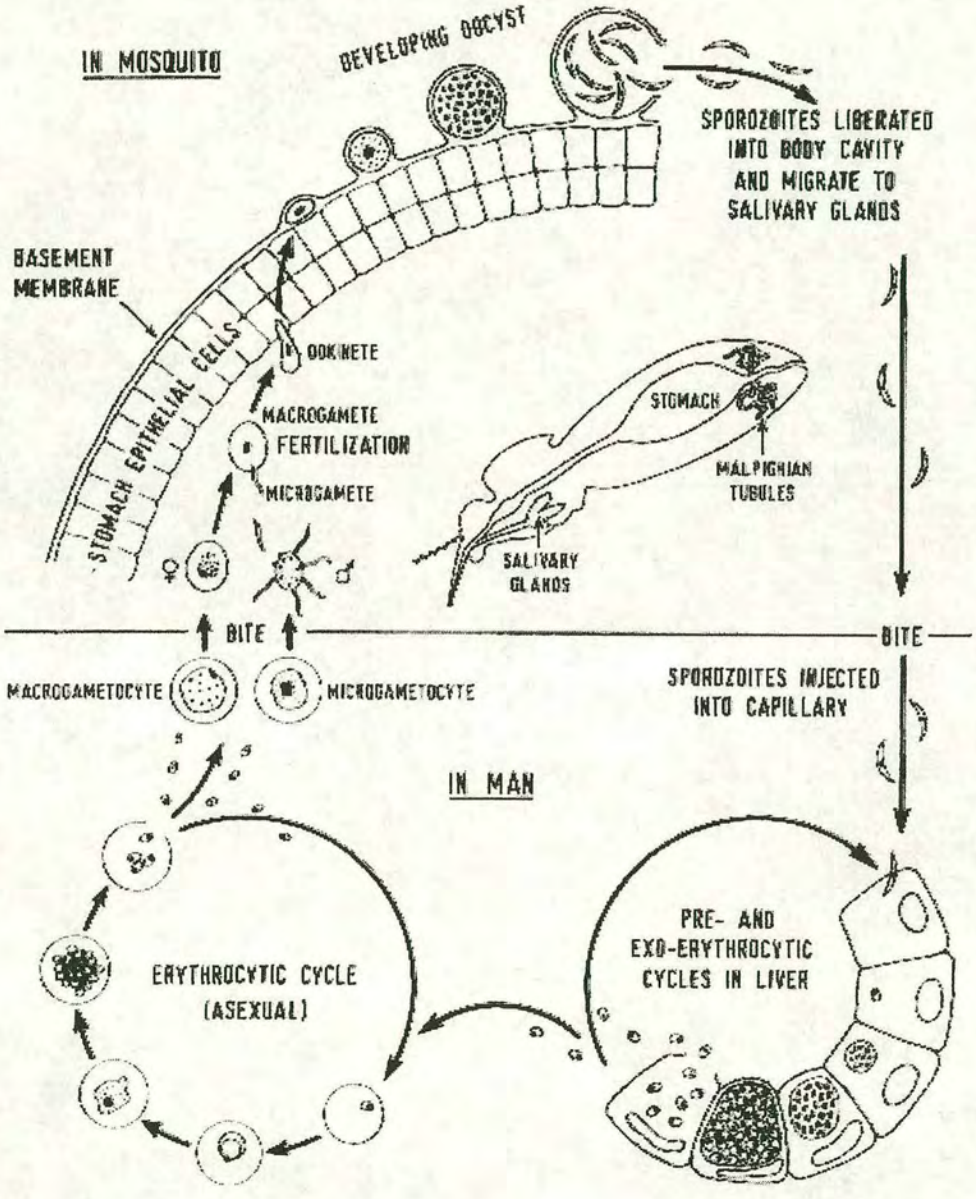


Figure 1.2: The innate immune response to malaria. It is hypothesised that when Mø come into contact with malaria Ag, IL-12 is produced to upregulate IFN-γ production by NK cells. IFN-γ acts synergistically with malaria antigens to upregulate TNF-α, IL-1 and IL-6 productions from Mø (adapted from (279)).

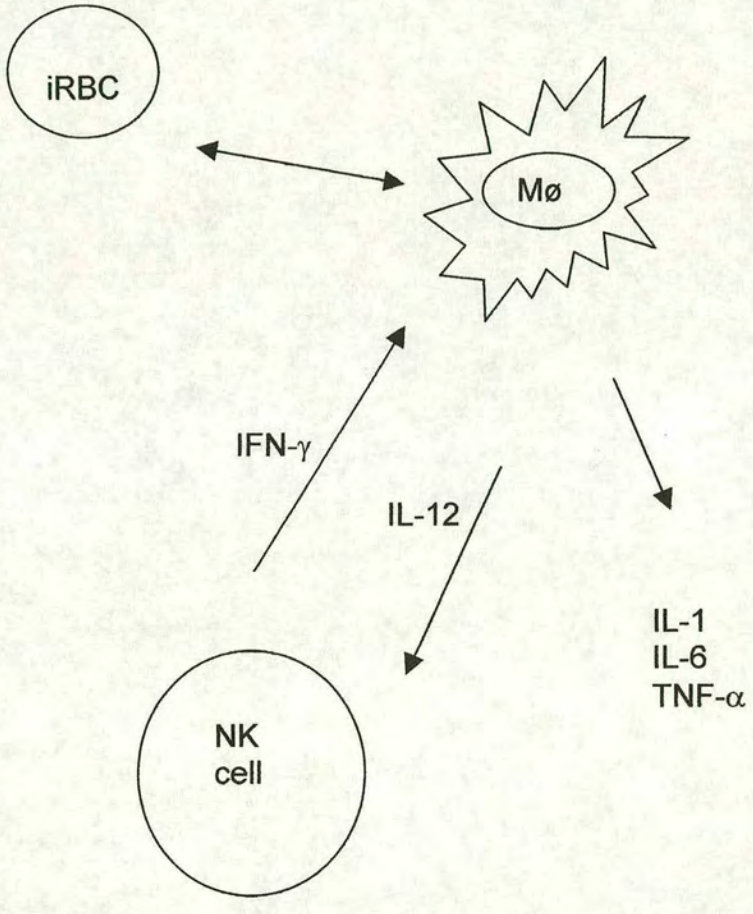


Figure 1.3: Primed individuals (naives and semi-immunes). It is hypothesised that CD4+ αβ+ cells interact with Ag presenting cells (APC) to proliferate and produce IFN-γ and IL-2. γδ+ cells bind malaria Ag in the presence of IL-2, and secrete IFN-γ. IFN-γ from both CD4+ αβ+ and γδ+ cells stimulates macrophages to produce increased amounts of inflammatory cytokines leading to pathology (adapted from (279)).

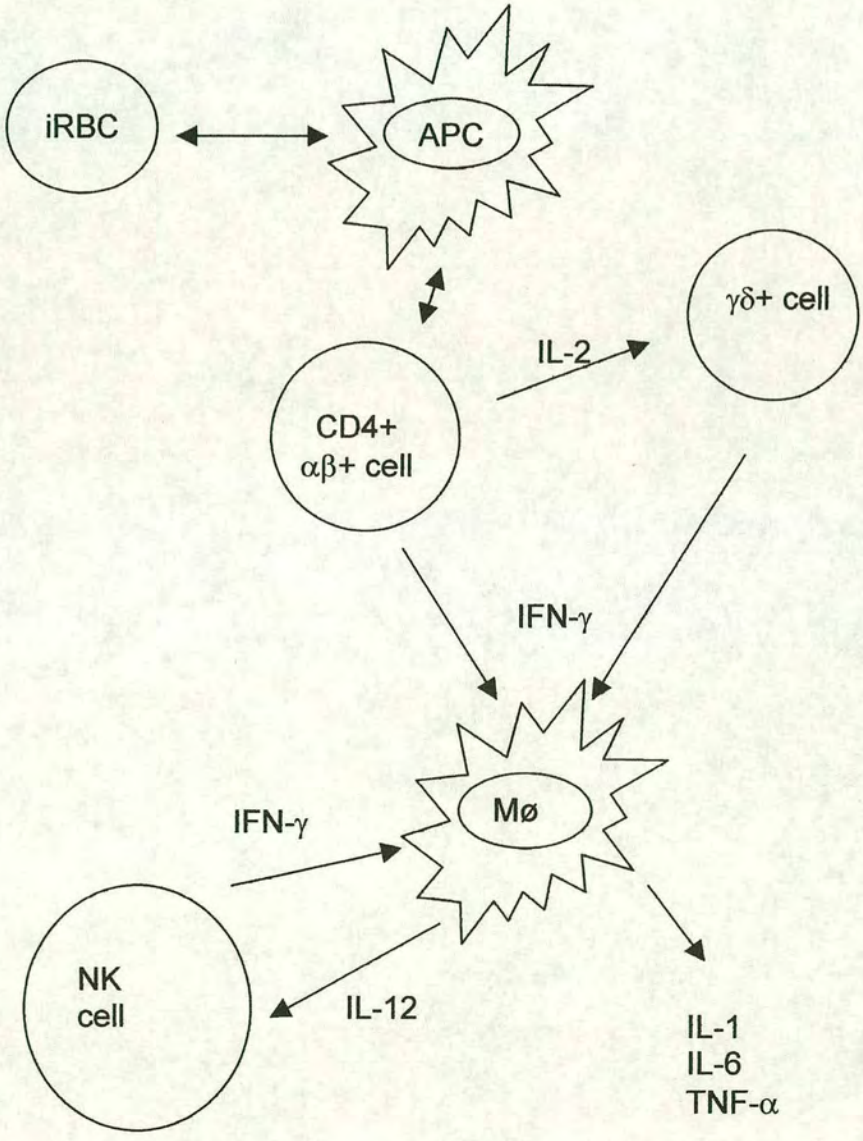
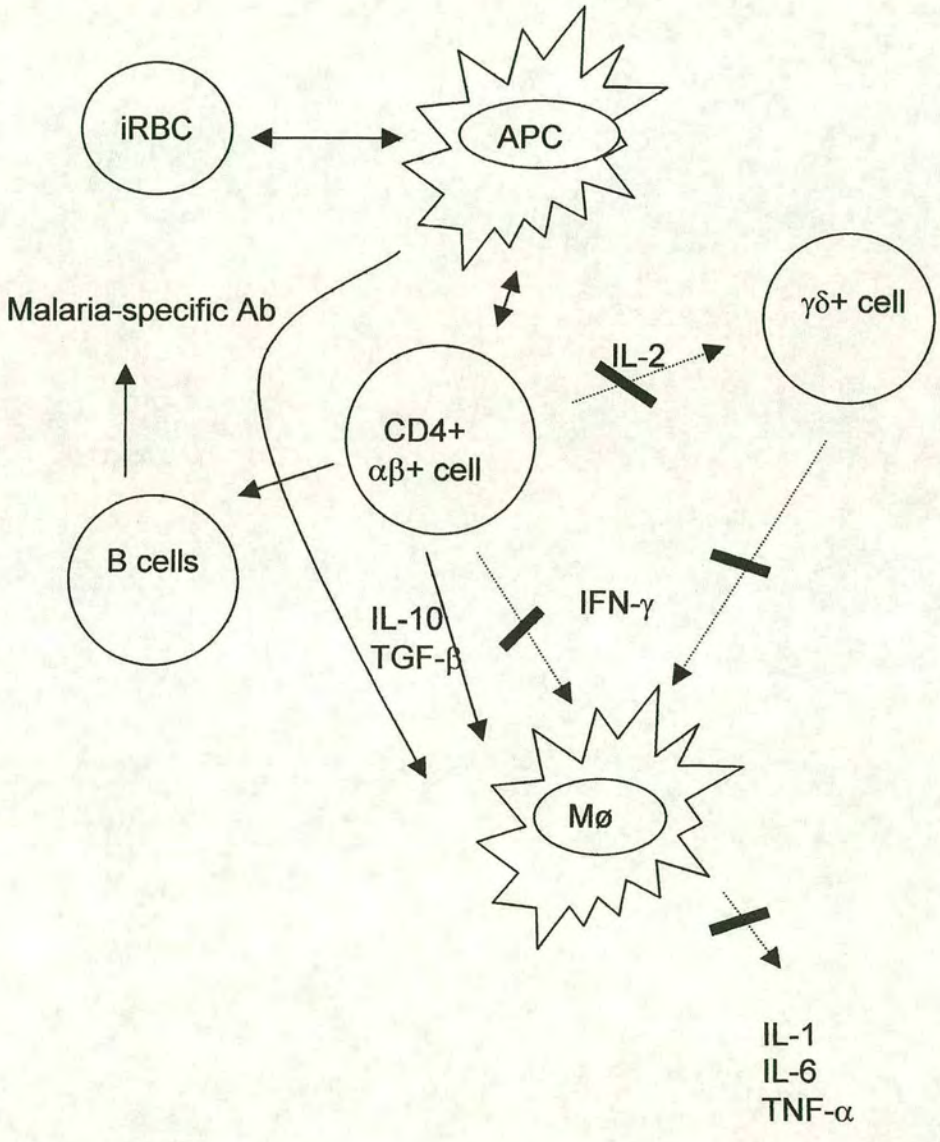


Figure 1.4: **Clinically immune individuals.** It is hypothesised that the inflammatory response is downregulated by the production of anti-inflammatory cytokines and memory T cells switch to a Th2-like, Ab-mediated response (adapted from (279)).



Chapter 2. Materials and methods

2.1 Subjects and serology

2.1.1 Study populations

A total of twenty-two malaria-naïve individuals living in Edinburgh, Scotland, UK were recruited for the various purposes of this study (table 2.1a). Most of the individuals had not travelled to a malaria-endemic country within the two years before blood samples were collected, and none of them have been infected with malaria.

Twenty donors were recruited from Dodowa, a small village about 40 miles inland from Accra, the capital city of Ghana (table 2.1b). Malaria transmission is highly endemic in this area (20 infective bites/year) with over 40% prevalence of parasitaemia and with the highest intensity of infection found in children under 5 years of age (5). Most of the adults do not develop clinical symptoms when infected with malaria. These individuals were designated malaria-immune individuals.

Twenty-one individuals were recruited from the greater Accra area (table 2.1c). Malaria transmission has been reported to be much lower in this area (29), and many adults have clinical symptoms when infected with malaria. This group was designated malaria-exposed, but not clinically immune.

Five additional naïve individuals were used as controls when the studies were carried out in Ghana. All of the individuals were taking anti-malarial prophylactics and were not infected with malaria at the time of blood collection (table 2.1d).

A questionnaire was completed for each donor, providing information on previous exposure to malaria and use of anti-malarial drugs. Tests for malaria parasitemia and Ab responses to malaria Ag were also done on most individuals (protocols described in sections 2.1.2 and 2.1.3). The questionnaire and serology data are presented in tables 2.1a-d and summarised in table 2.2.

2.1.2 Parasite detection

Thick and thin blood films from African donors were stained with Giemsa (BDH; Dorset, UK) and examined for the presence of malaria parasites. Donors were considered negative if no parasites were detected after 500 white blood cells had been counted.

In order to detect low levels of parasites, parasite DNA was detected by a polymerase chain reaction (PCR) method based on the multicopy 7H8/6 gene sequence (205). DNA was extracted from a small sample of blood by a method described by Foley *et al.* Briefly, 20 μl of whole blood were mixed with 500 μl of ice cold sodium phosphate, pH 8 (Sigma; Poole, UK) and centrifuged for 10 minutes at maximum speed in a picofuge. After the supernatant was discarded, the procedure was repeated twice more. The pellet was boiled with 50 μl of water for 10 minutes and then centrifuged for 10 minutes. The pellet was discarded and the supernatant used for PCR (103).

PCR conditions were optimised by Dr. Gillian Wagner in our laboratory (347). The primers were donated from Professor Allan Saul (Queensland Institute for Medical Research; Brisbane, Australia), and the sequences were 5'-ACATTATCATAATGAC(T)CCAGAACT-3' and 5'-GTTTCCAATAATTCTTTTTCTATC-3'. One microliter of extracted DNA was amplified in a 20 μl reaction mixture of 1X reaction buffer (500 mM KCl, 100 mM Tris-HCl (pH 9 at 25°C), 1% triton-x 100; Promega; Southampton), 0.5 U of Taq DNA polymerase (Promega), 4 mM $MgCl_2$ (Sigma), 75mM of each nucleotide (dATP, dCTP, dGTP, and dTTP; Boehringer-Mannheim; East Sussex, UK), and 100nM of each primer. The reaction was carried out for 35 cycles (94°C for 30 seconds, 55.8°C for 60 seconds and 72°C for 30 seconds) on a Hybaid thermocycler (Ashford, UK). PCR fragments were separated on ethidium bromide (EtBr; 0.5 μg/ml; Sigma)-stained, 2% agarose (Boehringer-Mannheim) gels run in 0.5X Tris-borate-EDTA (TBE) buffer, and visualised under ultraviolet light. Details of buffers and solutions are given in section in 4.2.3.

2.1.3 Detection of antibodies to malaria antigen

Plasma samples from all exposed and immune and most naïve donors were tested for anti-malarial antibodies by ELISA. Immulon IV microtitre plates (Dynex, Billingshurst, UK) were coated with an optimal concentration of soluble malaria Ag (described in section 2.2) diluted in Na_2CO_3/$NaHCO_3$ buffer (15mM Na_2CO_3, $NaHCO_3$, 0.02% NaN_3; all from BDH) and incubated overnight at 4°C. The plates were washed 3 times in phosphate-buffered saline (PBS) containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma). Plates were blocked for 3 hours at room temperature

(RT) with PBS containing 0.05% Tween 20 and 1% non-fat milk powder (blocking buffer) and washed as before. Plasma samples, diluted 1:1000 in blocking buffer, were added to duplicate wells, and incubated for 3 hours at RT. After another wash, 100 μl of rabbit anti-human IgG-horse radish peroxidase (Dako, High Wycombe, UK) were added to each well at a 1:1000 final dilution. The plates were incubated for 3 hours at RT, washed, and bound Ab was finally detected with an *o*-phenylenediamine (OPD; 100 μl/well for 10 minutes; Sigma) solution. The reaction was stopped with 2 M H_2SO_4 (BDH) and, optical density (OD) was measured at 492nm. Samples were designated positive for anti-malarial Ab if the OD was greater than the mean plus 2 standard deviations (SD) of the OD values of 22 control European plasma samples. Ab levels are expressed as a % of the value obtained for a pool of hyperimmune plasma from African adults tested on the same plate.

2.2 *In vitro* culturing of *P. falciparum* asexual stages

2.2.1 Parasite culture medium

1. Incomplete parasite culture medium
   RPMI-1640 medium (Gibco Life Technologies; Paisley, UK)
   25 mM hepes buffer (Sigma)
   0.15 % $NaHCO_3$ (ICN; Basingstoke, UK)
   5 μg/ml hypoxanthine (Sigma)
2. Complete parasite culture medium
   incomplete parasite culture medium
   10% heat-inactivated human O+ serum (Scottish Blood Transfusion Service; Edinburgh, UK)

2.2.2 Maintenance of *P. falciparum* cultures

Human O+ red blood cells (RBCs; Scottish Blood Transfusion Service) were infected with the 3D7 clone of *P. falciparum* parasite isolate NF54 (351) and cultured according to standard protocol (48). Briefly, parasites were continuously cultured at 2-4% hematocrit in complete parasite culture medium at 37°C in a humid, 5% CO_2-95% air atmosphere. Medium was changed daily and cultures were examined by Giemsa-stained thin blood smears. Cultures were grown to 6% parasitaemia and either used for

antigen preparation or passaged by diluting to 0.8% parasitaemia with fresh uninfected RBCs.

It has been previously reported that *Mycoplasma spp* can survive in long term *P. falciparum* cultures (292, 339) and that *Mycoplasma spp* have also been detected in certain malaria strains kept in Edinburgh (31). As mycoplasma are potent inducers of cytokines (292), cultures were routinely screened for mycoplasma using a commercial PCR kit (BioWhittaker; Wokingham, UK) and were confirmed to be free of contamination.

2.2.3 *P. falciparum* crude antigen preparation

Mature schizonts were separated from the rest of the culture on a 60% percoll gradient (Amersham-Pharmacia; Buckinghamshire, UK), washed twice in incomplete parasite culture medium, and resuspended at 10^8 schizonts/ml. Parasites were lysed by three freeze-thaw cycles in liquid nitrogen. The whole extract (PfSE) was used for cell culture experiments. The supernatant portion of the extract was used for serology (see section 2.1.3).

Optimal concentrations of PfSE were determined by titration in a lymphocyte proliferation assay (see section 2.5). Different batches of PfSE were standardised by using dilutions giving equivalent radioactive thymidine incorporation readings. A freeze-thaw preparation of uninfected RBCs (uRBC, 10^8/ml) was used as a negative control for PfSE. All preparations were aliquoted and stored at –80°C until required.

2.3 Other stimuli used in cell culture experiments

The following reagents were used to demonstrate the different kinetics of cellular immune responses and to compare these responses to the kinetics of the response to *P. falciparum* antigens.

1. 2 μg/ml of PHA (Sigma) was used as a T cell mitogen and as a positive control for normal cellular proliferation.
2. 10 μg/ml of LPS (Sigma) plus 10 ng/ml recombinant IFN-γ (TCS; Botolph Claydon, UK) were used as a positive control for IL-12 production.
3. 2 μg/ml of SEB (Sigma) was used as a superantigen and as a positive control for normal cellular proliferation.

4. 100 U/ml of PPD (Evans Medical Ltd.; Horsham, UK) was used as a classical T cell Ag.

5. Culture medium alone (CM) was used as a negative control for the stimuli.

6. 5 μg/ml of neutralising anti-interleukin-12 (αIL-12; R&D Systems; Abingdon, UK) was used to neutralise interleukin-12 (IL-12) produced by cell cultures.

7. 5 μg/ml of goat IgG (R&D Systems) was used as a negative control for αIL-12.

2.4 *In vitro* cell cultures

2.4.1 Cell culture medium

1. Incomplete cell culture medium
   - RPMI-1640 medium
   - 25 mM hepes buffer
   - 0.15% $NaHCO_3$
   - 0.4 mM L-glutamine
   - 100 U/ml penicillin (Sigma)
   - 0.1 mg/ml streptomycin (Sigma)
2. Complete cell culture medium
   - incomplete cell culture medium
   - 10% heat-inactivated human O+ serum

2.4.2 Separation of PBMCs

Venous blood was collected into heparinised tubes (1 U/ml of blood; Sigma) and diluted 2-fold with incomplete cell culture medium. Ten ml of diluted blood were layered onto 4 ml Lymphoprep® (Nycomed; Birmingham, UK) and centrifuged for 10 minutes at 400 g. PBMCs were collected from the interface, washed twice in incomplete cell culture medium, and resuspended at 10^6 cells/ml in complete cell culture medium for cell culture experiments.

2.5 Lymphocyte proliferation assays

2.5.1 Assay set-up

Cultures were set up in triplicate in round-bottomed 96-well microtitre plates (MacKay and Lynn; Edinburgh, UK). PBMCs were distributed at 10^5 cells/well in a total

culture volume of 200 μl with their corresponding stimuli and appropriate controls. All cell cultures were incubated at 37°C in a humid, 5% CO_2-95% air atmosphere for up to 9 days. Eighteen hours before harvest, cultures were pulsed with 1 μCi/well of radioactive [methyl-^{3}H] thymidine (Amersham-Pharmacia). Cultures were harvested in one of two ways. In the UK, cultures were harvested onto filter mats (EG&G Wallac; Milton Keynes, UK) using a Tomtec 96 Mach II harvester (EG&G Wallac). Scintillation fluid was melted onto the filter mats (Meltilex A; EG&G Wallac), and [methyl-^{3}H] thymidine incorporation was assayed by a 1450 Microbeta Trilux liquid scintillation and luminescence counter (EG&G Wallac). In Ghana, cultures were harvested onto glass fibre filters (Canberra-Packard; Pangbourne, UK) using a Filtermate 196 Cell Harvester (Packard). [methyl-^{3}H] thymidine incorporation was assayed by a Matrix 96 direct beta counter (Packard).

2.5.2 Data analysis

The geometric mean counts per minute (cpm) for triplicate wells were calculated and expressed as mean cpm. The stimulation index (SI) was calculated as the geometric mean cpm of cells stimulated with antigen/mitogen divided by the geometric mean cpm of cells stimulated with the corresponding control.

2.6 Measuring cytokines by enzyme-linked immunosorbent assay (ELISA)

2.6.1 Solutions used in ELISA

1. Coating buffer- PBS, pH 7.4
   - 9.6 g PBS
   - 1 L sterile distilled water
2. Blocking buffer (samples/controls, detection antibody, and enzyme diluted in this solution)
   - PBS, pH 7.4
   - 4% bovine serum albumin (BSA; Sigma)
3. Wash solution
   - PBS
   - 0.05% Tween 20

4. Substrate

10 mg OPD

6 ml 0.1 M citric acid (Sigma)

6.4 ml: 0.2 M Na_2HPO_4 (Sigma)

12.5 ml distilled water

20 μl H_2O_2

5. Stop solution

2 M H_2SO_4

2.6.2 Cell cultures

PBMCs were distributed at 10^6/well in flat-bottomed 24-well plates or in triplicate at 10^5/well in round-bottomed 96-well microtitre plates in a total of 1 ml and 200 μl respectively. Cultures were incubated for up to 8 days with their corresponding antigens, mitogens, or antibodies. Depending on the assay, 300-900 μl of culture supernatant were harvested at various timepoints and stored at -70°C until tested for cytokine levels.

2.6.3 Basic protocol for the sandwich ELISA

IFN-γ, IL-12 p70, IL-12 p40, and IL-10 were measured using the indirect, sandwich ELISA. Immulon 4, 96-well, flat-bottom microtitre plates were coated with 100 μl/well of mouse anti-human cytokine monoclonal Ab and incubated at RT overnight (for the complete list of Ab concentrations, refer to chapter 3). Plates were washed 4 times with wash solution, blocked with 200 μl/well blocking buffer for 1 hour at 37°C, and then washed 3 more times. Fifty microliters per well of recombinant cytokine standard (20,000 to 1 pg/ml) or culture supernatant were added to duplicate wells and incubated at RT for 2-3 hours. After 4 washes, 50 μl/well of biotinylated mouse anti-human cytokine monoclonal Ab were added and incubated for 1 hour at RT. After the plates were washed 6 more times, 100 μl/well of avidin-labelled horseradish peroxidase (HRP; Sigma) was added at 0.5 μg or 1 μg/ml solution for thirty minutes at RT. After a final 8 washes, plates were developed using OPD and H_2O_2 as substrate. After 15 minutes, the reaction was stopped with 25 μl/well of 2 M H_2SO_4, and absorbance was

read at 492 nm using an automated ELISA plate reader (Titretek Multiscan II; Life Sciences International; Basingstoke, UK)

2.6.4 Data analysis

Concentrations of cytokines were calculated from the standard curve given by the reference standard. Linear regression analysis was done and the best fit curve formula was used to calculate cytokine concentrations. The lowest level of detection (LLD) was determined as two standard deviations above the mean of the negative control (culture medium alone).

2.7 IL-12 p40 detection by reverse transcriptase polymerase chain reaction (RT-PCR)

2.7.1 Cell cultures

PBMCs were distributed at 10^6/well in a total of 1 ml of complete culture medium in flat-bottomed 24-well plates and cultured for up to 8 days with various stimuli. PBMCs were harvested, at various timepoints, into 15 ml conical tubes and washed twice with incomplete cell culture medium. Adherent cells in all cell cultures were detached from the wells with trypsin-EDTA (3.3X; Sigma) at 37°C for 15 minutes, inactivated with complete cell culture medium, and added to non-adherent cell suspensions. Cells were pelleted for immediate RNA extraction or stored at -70°C for future RNA extraction.

2.7.2 Basic protocol for qualitative RT-PCR

RNA was isolated using either a commercial RNA extraction kit or an assay based on guanidinium extraction (see Chapter 4 for complete details of the RT-PCR assay). RNA was extracted, resuspended in 50 μl of RNAse-free water and then treated with DNAse before using it for first strand synthesis. Complementary DNA (cDNA) was reverse transcribed from RNA with oligo-(dT) primer. Synthesis was run at 42 °C for 50 minutes and then stopped by raising the temperature to 70 °C for 15 minutes.

IL-12 p40 primers were synthesised by Oswel (Southampton, UK) based on published sequences. The sequences for IL-12 p40 and β-actin (a constitutively expressed gene used as a positive control) are described in table 4.1. One microliter of

cDNA was amplified in a 10 μl reaction mixture of 1X buffer, 0.5 U Taq DNA polymerase, 2 mM $MgCl_2$, 200 μM dNTP mix, and 1μM each cytokine primer. The reaction was carried out for 35 cycles (94°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute) on Hybaid thermocycler. PCR fragments were separated on EtBr-stained, 1.5% agarose gels run in 0.5X TBE buffer and visualised under ultraviolet light.

Fidelity of the amplified PCR product was confirmed to be β-actin or IL-12 p40 by using a commercial sequencing kit and by then comparing sequences to the GEN-EMBL database.

2.8 Cell phenotyping by flow cytometry

2.8.1 Cell cultures

PBMCs were distributed at 1x or $2x10^6$/well in a total of 1 or 2 ml culture medium respectively in flat-bottomed 24-well plates. Each culture had a total of 2 or $4x10^6$ cells (except for immune individuals where there were a limited number of PBMCs and cell numbers were as low as $3.5x10^6$/10 ml whole blood). After cells were cultured for 3 or 7 days, PBMCs were harvested into 15 ml conical tubes and washed twice with PBS supplemented with 0.1% NaN_3 and 0.1% BSA (FACs PBS) and resuspended in FACs PBS at $2x10^6$/ml.

2.8.2 Monoclonal antibody staining

Cells were stained on days 0, 3 and 7 for analysis of surface markers by 3 colour flow cytometric analysis. Cells were equally distributed into round-bottomed 96-well microtitre plates. Each well contained 20 μl of each of 3 different antibodies. Cells were incubated at 4°C for 40 minutes. Cells were washed twice and then transferred to 5 ml round-bottomed tubes in 300 μl of FACs PBS for analysis. One cell aliquot was taken for an unstained sample control, and another cell aliquot was used for determining cell viability by trypan blue (Sigma) exclusion and absolute cell counts by haemocytometry.

All antibodies were directly conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE), or tricolour (TRI). Anti-CD20, anti-TcR αβ and anti-TcR γδ (all from TCS; Botolph Claydon, UK) were all FITC-labelled. Anti-CD45RA, anti-CD45RO, anti-CD4, and anti-CD8 were either FITC- or PE-labelled (all from TCS). Anti-CD56 was PE-labelled and anti-CD3 was TRI-labelled (both from TCS). All antibodies were used

at a final concentration of 0.2 μg per staining combination with the exception of anti-CD8 which was used at 0.05 μg per staining combination. Up to 9 different staining combinations were done, depending on the number of cells available, in order to identify different cell subsets. For the different sets of staining combinations and which cells they identify, refer to section 7.2.

2.8.3 Data analysis

Cell phenotyping was performed by flow cytometry (FACscan; Becton-Dickinson; London, UK). Ten thousand to 20,000 events were acquired per sample. Red blood cells and dead cells were gated out, and single stains were used to set the compensation for the fluorescence. Resting cells and lymphoblasts were gated separately, detected by forward scattered light (FSC) versus side scattered light (SSC), and analysed for the different staining combinations. Cell quest software package (Becton-Dickinson) was used for data analysis. Percentages and absolute numbers of cell subsets were calculated.

2.9 Detection of IFN-γ by intracellular cytokine staining (ICS)

2.9.1 Solutions used in ICS

1. Staining buffer
   PBS
   1% fetal calf's serum (Sigma)
   0.1% NaN_3
2. Fixation buffer
   PBS
   4% paraformaldehyde (BDH)
   pH7.5
3. Permeabilisation buffer
   Staining buffer
   0.3% saponin (BDH)

2.9.2 Cell cultures

PBMCs were distributed at $2x10^6$/well in flat-bottomed 24-well plates in a total of 2 ml. A total of $6x10^6$ cells (i.e. 3 wells) were used for each stimulus and all cultures were incubated for 7 days. Eighteen hours before harvest, 3 μg of Brefeldin A (Sigma) was added per 1 ml of culture in order to stop Golgi transport of intracellular IFN-γ. PBMCs were then harvested into 15 ml conical tubes, washed twice with PBS and resuspended in staining buffer at 10^6/ml.

2.9.3 ICS protocol

Cells were first stained for surface markers as detailed in section 2.8.2. After one wash with PBS, cells were resuspended in 300 μl fixation buffer and incubated at RT in the dark for 15 minutes. Cells were washed again and then resuspended in 300 μl permeabilisation buffer which contained 1-2 μg of either FITC labelled anti-IFN-γ (TCS) or PE-labelled anti-IFN-γ (Becton-Dickinson). Cells were incubated at 4°C in the dark for 30 minutes. Cells were washed once in permeabilisation buffer and resuspended in 0.5 ml of PBS for flow cytometry analysis as described in section 2.8.3.

2.10 Statistical analysis

All statistical analysis was performed with Microsoft excel (Seattle, Washington, USA) or Minitab 12 (Microsoft) software packages. Data was tested for normal distribution by the Ryan-Joiner normality test. Differences between groups were assessed by Student's t test on normal or log-transformed data. Differences in response to different treatments within groups, were assessed by paired t tests comparing treated and control cells from the same donors. The non-parametric Mann Whitney U test was used for severely skewed (non-normal even after log transformation) data, although McGuinness *et al.* demonstrated that normal parametric tests are still quite robust on highly skewed data (223). Chi-squared (χ^2)analysis was performed to compare responders versus non-responders. All correlations were done with the Pearson correlation test. For complete descriptions of these tests, refer to Kirkwood (1990) (174).

Table 2.1a: Summary of questionnaire data from naïve individuals living in Edinburgh, UK.

ID #	Sex	Age	Blood	Travel	When	Where	Malaria	Antimalarials	Type	Crude Ag	Ab
N1	F	39	Sep-97	N	N/A	N/A	N	N/A	N/A	ND	ND
N2	F	24	Sep-97	Y	Aug-96	Zimbabwe	N	Y	Larium	-	0.05
N3	M	36	Nov-97	N	N/A	N/A	N	N/A	N/A	-	0.05
N4	M	21	Nov-97	N	N/A	N/A	N	N/A	N/A	ND	ND
N5	M	38	Nov-97	N	N/A	N/A	N	N/A	N/A	ND	ND
N6	M	25	Jul-98	Y	Jul-97	Indonesia	N	Y	Larium	ND	ND
N7	F	26	Jul-98	N	N/A	N/A	N	N/A	N/A	ND	ND
N8	M	35	Jul-98	N	N/A	N/A	N	N/A	N/A	-	0.04
N9	M	20	Jul-98	N	N/A	N/A	N	N/A	N/A	ND	ND
N10	F	31	Jul-98	N	N/A	N/A	N	N/A	N/A	ND	ND
N11	F	22	Jul-98	N	N/A	N/A	N	N/A	N/A	-	0.04
N12	M	41	Oct-98	Y	Dec-97	Ivory Coast	N	Y	Chloroquine/Palludrine	-	0.04
N13	M	27	Oct-98	N	N/A	N/A	N	N/A	N/A	ND	ND
N14	F	31	Oct-98	N	N/A	N/A	N	N/A	N/A	-	0.06
N15	M	24	Oct-98	N	N/A	N/A	N	N/A	N/A	-	0.05
N16	F	33	Nov-98	Y	Jan-98	Kenya	N	Y	Chloroquine/Palludrine	-	0.08
N17	F	30	Nov-98	N	N/A	N/A	N	N/A	N/A	-	0.05
N18	M	34	Nov-98	Y	Apr-98	Kenya	N	N	N/A	-	0.07
N19	M	29	Nov-98	N	N/A	N/A	N	N/A	N/A	-	0.05
N20	M	24	Feb-99	N	N/A	N/A	N	N/A	N/A	ND	ND
N21	F	21	Jun-99	N	N/A	N/A	N	N/A	N/A	ND	ND
N22	M	24	Jun-99	N	N/A	N/A	N	N/A	N/A	ND	ND

Sex- Male (M) or female (F).
Blood- Date when blood sample was taken for the study.
Travel- Have you traveled in a malaria endemic country within the 2 years prior to your blood being taken?
When- When did you travel?
Where- Where did you travel?
Malaria- Did you contract malaria?
Antimal- Were you taking antimalarials?
Type- What kind of antimalarials were you taking?
Crude Ag- Positive (+) or negative (-) Ab response to crude malaria Ag measured by ELISA.
Ab- OD value given as a ratio of average OD value of Brefet sera, a highly immune sera.
N/A- Not applicable.
ND- Not done.

Table 2.1b: Summary of questionnaire data from exposed individuals living in Accra, Ghana.

ID #	Sex	Age	Travel	Mal/yr	Last At	Confm	Severity	Treat	Antimal	Film	PCR	Crude Ag	Ab
E1	M	37	Y	N	96	N	Mi	Y	N	-	-	+	0.27
E2	M	28	N	Y	Jan-98	N	Mi	Y	N	-	-	+	0.31
E3	M	40	Y	N	Jun-96	Y	Mi	Y	N	-	-	-	0.23
E4	F	34	Y	Y	?	Y	Mi	?	N	-	-	+	0.47
E5	F	25	N	Y	96	Y	Ma	Y	N	-	-	+	0.26
E6	M	26	N	Y	Oct-97	N	Mi	Y	N	-	-	+	0.26
E7	M	28	N	N	96	N	Mi	Y	N	-	-	+	0.35
E8	M	26	N	Y	Dec-97	Y	Ma	Y	N	-	-	-	0.17
E9	M	31	N	Y	Dec-97	Y	Ma	Y	N	-	-	+	0.86
E10	M	50	N	?	Mar-94	N	Ma	Y	N	-	-	+	0.85
E11	M	46	Y	Y	Jan-98	N	Mi	Y	N	-	-	-	0.13
E12	M	46	Y	Y	Mar-98	Y	Mi	Y	N	-	-	+	0.54
E14	M	60	N	Y	Jan-98	Y	Mi	Y	N	-	-	+	0.73
E15	M	48	Y	N	Oct-97	Y	Ma	Y	Y*	-	-	+	0.60
E16	M	29	N	Y	Dec-97	Y	Mi	Y	Y*	-	-	+	0.43
E17	F	21	N	N	Feb-96	Y	Mi	Y	Y*	-	-	+	0.26
E18	M	38	N	N	Jun-96	Y	Mi	Y	Y*	-	-	+	1.01
E19	M	41	Y	N	Sep-93	Y	Ma	Y	N	-	+	-	0.20
E20	M	52	N	Y	97	N	Mi	Y	Y*	-	-	+	0.35
E21	M	28	N	Y	Sep-97	Y	Mi	Y	Y*	-	-	-	0.20
E22	M	29	N	?	Jun-97	?	Ma	Y	N	-	-	-	0.24

Sex- Male (M) or female (F)
Travel- Have you traveled within the 2 years prior to your blood being taken?
Mal/yr- Do you get malaria yearly?
Last At- When was your last attack?
Confm-Was it confirmed by blood film?
Severity- How severe was the illness? Major (Ma) or minor (Mi). Time off work was considered a major illness.
Treat- Were you treated for this last episode of malaria?
Antimal- Do you use antimalarials regularly? *They really mean no, because the date they gave when they last used antimalarials was when they had malaria.
Film- Malaria status confirmed by blood film at the time of bleed.
PCR- Malaria status confirmed by PCR at the time of bleed.
Crude Ag- Positive (+) or negative (-) Ab response to crude malaria Ag measured by ELISA.
Ab- OD value given as a ratio of average OD value of Brefet sera, a highly immune sera.

Table 2.1c: Summary of questionnaire data from immune individuals living in Dodowa, Ghana.

ID #	Sex	Age	Travel	Mal/yr	Last At	Confm	Severity	Treat	Antimal	Film	PCR	Crude Ag	Ab
I1	M	45	N	Y	Jun-98	N	Mi	Y	N	-	+	+	1.27
I2	F	46	N	Y	Oct-98	N	Mi	Y	N	-	-	+	0.93
I3	F	30	N	Y	Nov-98	N	Mi	Y	N	+	-	+	0.65
I4	F	30	N	Y	Jul-98	N	Mi	Y	N	-	-	+	1.16
I5	F	33	N	Y	Jan-99	N	Mi	Y	N	+	-	+	1.14
I6	F	40	N	Y	Oct-98	N	Mi	Y	N	-	-	+	0.46
I7	F	34	N	Y	Jul-98	Y	Ma	Y	N	-	-	+	0.88
I8	F	33	N	Y	Feb-99	N	Ma	Y	N	-	+	+	0.45
I9	F	31	N	Y	Jan-99	N	Mi	Y	N	-	+	+	0.29
I10	M	30	N	Y	Feb-99	N	Ma	Y	N	-	-	+	0.42
I11	M	38	N	Y	Oct-98	N	Mi	Y	N	-	+	+	0.97
I12	M	32	N	Y	Sep-98	N	Ma	Y	N	-	-	+	0.93
I13	F	34	N	Y	Nov-98	N	Mi	Y	N	-	-	+	0.74
I14	F	47	N	Y	Jan-99	N	Mi	Y	N	-	+	+	1.29
I15	M	38	N	Y	Feb-99	N	Ma	Y	Y	-	-	+	0.94
I16	F	30	N	Y	Feb-99	N	Mi	Y	N	-	+	+	1.43
I17	M	31	N	N	Sep-98	N	Mi	Y	N	-	-	+	1.44
I18	M	30	N	Y	Dec-98	N	Ma	Y	N	-	-	+	0.60
I19	M	30	N	Y	Jan-99	N	Mi	Y	N	-	-	+	1.07
I20	F	44	N	Y	Feb-99	N	Mi	Y	N	-	-	+	0.24

Sex- Male (M) or female (F)
Travel- Have you traveled within the 2 years prior to your blood being taken?
Mal/yr- Do you get malaria yearly?
Last At- When was your last attack?
Confm-Was it confirmed by blood film?
Severity- How severe was the illness? Major (Ma) or minor (Mi). Time off work was considered a major illness.
Treat- Were you treated for this last episode of malaria?
Antimal- Do you use antimalarials regularly?
Film- Malaria status confirmed by blood film at the time of bleed.
PCR- Malaria status confirmed by PCR at the time of bleed.
Crude Ag- Positive (+) or negative (-) Ab response to crude malaria Ag measured by ELISA.
Ab- OD value given as a ratio of average OD value of Brefet sera, a highly immune sera.

Table 2.1d: Summary of questionnaire data from naive individuals used as controls when studies were carried out in Ghana.*

ID #	Sex	Age	Blood	Travel	When	Where	Malaria	Antimalarials	Type	Crude Ag	Ab
C1	F	41	Mar-98	Y	Mar-98	Ghana	N	Y	Chloroquine/Palludrine	ND	ND
C2	F	39	Mar-98	Y	Mar-98	Ghana	N	Y	Chloroquine/Palludrine	ND	ND
C3	F	25	Mar-98	Y	Mar-98	Ghana	N	Y	Chloroquine/Palludrine	ND	ND
C4	M	39	Mar-98	Y	Mar-98	Ghana	N	Y	Larium	ND	ND
C5	F	25	Mar-99	Y	Mar-98	Ghana	N	Y	Chloroquine/Palludrine	-	0.06

Sex- Male (M) or female (F).
Blood- Date when blood sample was taken for the study.
Travel- Have you traveled in a malaria endemic country within the 2 years prior to your blood being taken?
When- When did you travel?
Where- Where did you travel?
Malaria- Did you contract malaria?
Antimal- Were you taking antimalarials?
Type- What kind of antimalarials were you taking?
Crude Ag- Positive (+) or negative (-) Ab response to crude malaria Ag measured by ELISA.
Ab- OD value given as a ratio of average OD value of Brefet sera, a highly immune sera.
ND- Not done.
*All of the control naïve individuals were confirmed negative for malaria infection by blood smear and PCR.

Table 2.2: Summary details of all blood donors.

	Naive (UK)	Exposed (Accra)	Immune (Dodowa)
N	22	21	20
Age range (years)	20-41	21-60	30-47
Blood film +	0 (0%)	0 (0%)	2 (10%)
PCR +	0 (0%)	1 (5%)	6 (30%)
Antibody +	0/11 (0%)	15/21 (71%)	20 (100%)
Mean Ab* (SEM)	0.05 (0.004)	0.41 (0.06)	0.87 (0.08)
Ab Range	0.04 – 0.08	0.13 – 1.01	0.24 – 1.44
Confirmed malaria[#]	0/22 (0%)	13/21 (62%)	1/20 (5%)

*OD values are expressed as a ratio of average OD value of Brefet sera, a highly immune sera.

[#] Confirmed clinical malaria in the past 5 years.

Chapter 3: Optimisation of enzyme-linked immunosorbent assay (ELISA) for measuring cytokine concentrations in cell culture supernatants

3.1 Introduction

The introduction of ELISA in 1971 by Engvall and Perlmann was a significant technical advance in the measurement of protein molecules (87-89). Although there were other methods already available for measuring and detecting antibodies and antigens such as radioactive immunoassays, none surpassed ELISA in its ease and simplicity. Overall, ELISA is much simpler, faster, requires less special equipment and its reagents are stable over a longer period of time than other protein detection assays.

Engvall and Perlmann initially used ELISA for the detection and quantification of immunoglobulin. The scope of the assay has since then become much broader, and it now has numerous uses in clinical diagnostics and in analytical research. ELISAs are used not only for measuring Ab levels, but also for detecting and quantifying antigens of infectious agents (68, 167), and more importantly for the purpose of this thesis, for measuring proteins such as cytokines.

3.1.1 The sandwich ELISA

The primary type of ELISA which is used to measure cytokine levels is the sandwich ELISA. In the sandwich ELISA (41, 170, 244, 270, 344), bound Ab first captures the cytokine and is then detected (hence, "sandwiched" between two antibodies) by another Ab recognising a different epitope (figure 3.1). Although the method is quick and relatively simple, care needs to be taken in the conditions.

3.2 Principles of assay optimisation

3.2.1 Choice of solid phase

There are a number of solid phase supports which are used, and these are divided into two general groups, high capacity and low capacity (how much protein they can bind) (167-169, 342). Although high capacity materials can bind much higher levels of protein and still be specific with crude coating material (170), the majority of researchers use low capacity materials which give low levels of nonspecific binding.

Polystyrene plates versus latex beads or tubes is the low capacity material of choice, because a large number of samples can be tested quickly. But care still must be taken with microtitre plates because there can be batch to batch variation and because the outer wells do not always bind protein as well as the inner plates (169, 342).

3.2.2 Coating

Incubation time, temperature, and buffer pH must all be optimised for the best sensitivity and specificity in each step of the assay. In particular, adsorption of coating Ab is said to increase with an increase in incubation time and temperature until a plateau is reached (342). But prolonged times at higher temperatures can also lead to a loss in function of the coated material (169). For some coating antibodies, the pH of the buffer can influence coating efficiency (167, 257), while for others it makes very little difference (169). Plates are typically coated overnight at 4°C with PBS pH 7.2-7.6 as the coating buffer.

The type and quality of coating and detecting antibodies are also important aspects of how sensitive and specific the assay can be (185, 313). It is important to have highly purified antibodies whether they are monoclonal or polyclonal, although monoclonals are usually used, because they have a reproducible affinity for a given epitope.

The method of coating, either direct or indirect, also makes a difference in binding efficiency (167, 168). The direct method, of passive adsorption of Ab onto the plate, is by far the most common method. But it has been shown that there can be a significant loss in function because of desorption when the Ab is not bound tightly enough and because of conformational changes of the Ab once bound (42, 342).

Concentration of all reagents is the most important aspect of each step in the ELISA. If the concentration of the coating Ab is too high, antibodies will tend to bind to each other, while if the concentration is too low, there can be nonspecific binding of detector Ab to the plate (168, 257). The concentration of each substance can be determined by titration for each type of experiment.

3.2.3 Sample conditions

There is specific binding of test samples to the capture Ab as well as nonspecific binding to the plate or Ab (169, 342). Blocking proteins, such as BSA or skimmed milk, are often added to reduce background noise from nonspecific binding. These proteins bind to vacant protein-binding sites on the plate. There are a maximum number of sample molecules that can specifically bind to the capture Ab. There tend to be weak affinity interactions between sample and Ab when concentrations are too high (168). Reducing sample volume helps increase binding efficiency.

3.2.4 Detector antibody

Again choice of buffer, temperature, incubation time and most of all, concentration (preferably used in slight excess) need to be standardised for the detecting Ab as for the coating Ab and sample. The specificity of the detector Ab is crucial and cross-reactions need to be minimised (167, 168).

Enzyme conjugation is also an important consideration. Detector antibodies are often directly labelled with enzyme, making the assay one step simpler. However, use of a second Ab or an avidin-biotin system allows this reagent to be used in many different assays (167, 168, 270). Biotinylated antibodies can bind to avidin-linked enzymes specifically with high affinity which gives the assay a higher sensitivity (185, 270).

3.2.5 Choice of enzyme and substrate

High turnover rate, low cost, reproducible quality, stability and absence of natural enzymes in test samples are all factors which need to be considered. Colourimetric methods are used more often over flourimetric methods because of product stability and quick, inexpensive equipment for measuring colour (257, 270). Horseradish peroxidase, beta-galactosidase, and alkaline phosphatase are the three main enzymes used for colourimetric methods. It has been shown that horseradish peroxidase has much higher activity and is less expensive, although chromogens are thought to be carcinogenic and need to be handled with care (269, 342, 345).

3.2.6 Data analysis

There is no set way of presenting ELISA data. It is often satisfactory just to define a cut-off value for positive samples (129, 167, 257). But even when looking for a simple positive or negative result in qualitative analysis, it is still necessary to optimise the assay and run proper controls.

As in this report, quantitative analysis needs to be done when comparing the amount of specific protein between different samples. This analysis is often done by reading from a standard curve. Three factors must be considered when using reference standards. First, standards must have the highest purity and stability so that reproducible results can be obtained each time the assay is run (126, 257). It is also advisable that standards are diluted in the same diluent as test samples (257). Second, the shape of the reference curve is also important. Because ELISA have a narrow range (the upper limit is 2-2.5 for optical density readings), it is better to have a very steep curve over fewer log dilutions than to have a shallow curve over a larger range of dilutions (167, 168). Finally, a lower limit of detection needs to be established for each assay. Typically the lower limit is defined as some formulation of the background value.

3.2.7 Conclusions

In conclusion, ELISA is a very useful tool to measure proteins quantitatively. Because of the widespread use of this assay, many companies have now developed high quality antibodies, enzymes, etc. for use in ELISA as well as complete ELISA kits. But even though materials have improved, it can be seen from the discussion above that care must still be taken when optimising the conditions of a particular assay. All of the materials for cytokine detection in this report were from companies which had previously developed, standardised, and purified them themselves. Regardless, assay parameters still had to be optimised for maximum specificity and sensitivity for each cytokine.

3.3 Optimisation of ELISA for measuring cytokine responses to *P. falciparum* antigen *in vitro*

Monoclonal antibodies to IFN-γ, IL-12 p70, IL-12 p40 and IL-10 were coated onto the solid phase (microtitre plates), and plates were incubated with cell culture

supernatants. Bound cytokine was "sandwiched" by a biotinylated monoclonal Ab and detected by avidin-linked HRP and OPD/H_2O_2. Each step was optimised for the best sensitivity and specificity to cytokine. The complete protocol and concentrations of reagents for each cytokine measured are found in tables 3.1 and 3.2

3.3.1 Solid phase

Immulon 4, 96-well, polystyrene plates were chosen for their ability to bind high levels of protein (Ab). They are specially designed for Ab adsorption and reproducibility between assays. Outer wells were never used, and plates were never stacked higher than 6 plates.

3.3.2 Reagent concentrations

3.3.2.1 Coating and detector antibodies

Coating and detector monoclonal antibodies had been highly purified by Ab affinity purification. Optimal concentrations of coating and detector antibodies were determined by checkerboard titration (168, 170, 345). Five dilutions of recombinant human cytokines were used to test varying concentrations of both antibodies (10, 5, 1, 0.5, and 0.1 ng/ml for IFN-γ, IL-12 p70 and IL-10 and 10, 1, 0.1, 0.01 and 0.001 ng/ml for IL-12 p40). Three of four dilutions (10, 5, 2, and 1 μg/ml for IFN-γ, IL-12 p70 and IL-10 and 4, 2 and 1 μg/ml for IL-12 p40) of the coating monoclonal Ab were tested with 3 dilutions (1, 0.25, and 0.05 μg/ml for IFN-γ, IL-12 p70 and IL-10 and 0.3, 0.15 and 0.075 μg/ml for IL-12 p40) of the detector monoclonal Ab. The concentrations which gave the widest range of OD readings (steepest standard curve) were 2 μg/ml of coating Ab for IFN-γ, IL-12 p70 and IL-10 and 4 μg/ml for IL-12 used with 0.05 μg/ml of detector Ab for IFN-γ and IL-10, 0.25 μg/ml for IL-12 p70 and 0.3 μg/ml for IL-12 p40 (table 3.2 and figures 3.2-3.3).

3.3.2.2 Standard and sample volume

Fifty and 100 μl volumes of recombinant human cytokine standards were tested in order to determine if there was a difference in the amount of sample which could be bound. There was little difference between the OD readings, thus 50 μl was chosen for economy of reagents (figure 3.4).

3.3.2.3 Enzyme and substrate detection system

HRP was chosen because it could be used with more than one biotinylated detecting Ab. It was also chosen because avidin-HRP with substrate OPD is one of the most sensitive, rapid and least expensive systems to use. Two or three dilutions of HRP were tested: 2.5, 1 and 0.5 μg/ml. The optimal concentrations were 0.5 μg/ml for IFN-γ and IL-12 p70 and 1 μg/ml for IL-10 and IL-12 p40 (figure 3.5).

.3.3 Miscellaneous protocol changes

All of the following optimisation steps were done to identify the lowest level of detection of cytokine and to reduce background without compromising the assay (Not all of the assays were performed on the IL-12 p40 ELISA as this assay was optimised after the other cytokine ELISAs. Many of these protocol changes were already assumed to be optimal).

3.3.3.1 Blocking step

A blocking step was done for 1 hour at 37°C instead of the recommended 1 hour at room temperature (figure 3.6).

3.3.3.2 Dilution medium

Four percent bovine serum albumin (BSA) was compared to 1% skimmed milk as blocking buffer and diluent for detecting Ab and HRP. Four percent BSA was used, because 1% skimmed milk gave lower OD readings over the entire range for IFN-γ and IL-12 p70 (figure 3.7).

3.3.4 Data analysis

Plates were read at 492 nm with an automated spectrophotometer. Each sample and standard was tested in duplicate and the arithmetic mean was taken. Concentrations of cytokine were calculated from the standard curve. Linear regression analysis was done and the best fit curve formula was used to calculate cytokine concentrations (figure 3.8). The LLD was determined as 2 SDs above the mean of the

negative control (culture medium alone or PBS). Representative data for cytokine production from PHA- or LPS-stimulated PBMCs are shown in figure 3.9.

3.4 ELISA: the best way to measure cytokines?

The study of cytokines is a rapidly growing field with new knowledge being accumulated almost daily. Measuring cytokine levels will undoubtedly become a standard requirement in clinical diagnosis. It is important to have tools which accurately portray the cytokine profile. The choice of assay depends on the amounts of cytokine produced. ELISAs can measure pg/ml amounts of cytokine. But if concentrations are much lower (e.g. IL-4 (75)), then PCR-based methods may be needed.

In this study, IFN-γ levels in test samples could be reliably measured by ELISA. However, levels of IL-12 p40 and IL-10 were almost always close to the LLD, and results were thus more difficult to interpret (see Chapter 6). RT-PCR methods were evaluated for IL-12 (see Chapter 4), but this method was also far from ideal. In some cases, neutralisation assays may be the most appropriate method of demonstrating cytokine production (e.g. αIL-12 Ab, see Chapter 6). Thus, methods other than ELISA should also be used in conjunction to measure cytokines. It has been found that some cytokines are only detectable by RT-PCR and not by ELISA and that significant decreases in cytokine production can be detected with ICS but not with ELISA (92, 357).

Because measuring cytokines by ELISA is the simplest method, there are two main factors which must be considered when using ELISAs. First, it must be noted that there are a number of naturally occurring molecules that inhibit cytokine detection and therefore skew results. It has been shown by a number of researchers that molecules such as alpha-2-macroglobulin, soluble receptors, and autoantibodies bind to cytokines, although most commercial companies have taken this factor into consideration (4, 58, 60, 124, 322, 359). Second, and more importantly, there is a lot of variation between commercial standards. Three separate studies demonstrated that there was a huge difference in standard and sample values between commercial kits (68, 203, 255). No two kits gave the same values. These studies emphasise the need to produce international standards for cytokine assays, especially if comparisons need to be revalidated between laboratories. Until then, standards need to be carefully evaluated

before they are used, and internal consistency must be ensured by using the same reagents and protocols throughout an experiment or a series of experiments.

Figure 3.1: Schematic representation of the sandwich ELISA used for measuring cytokine levels.

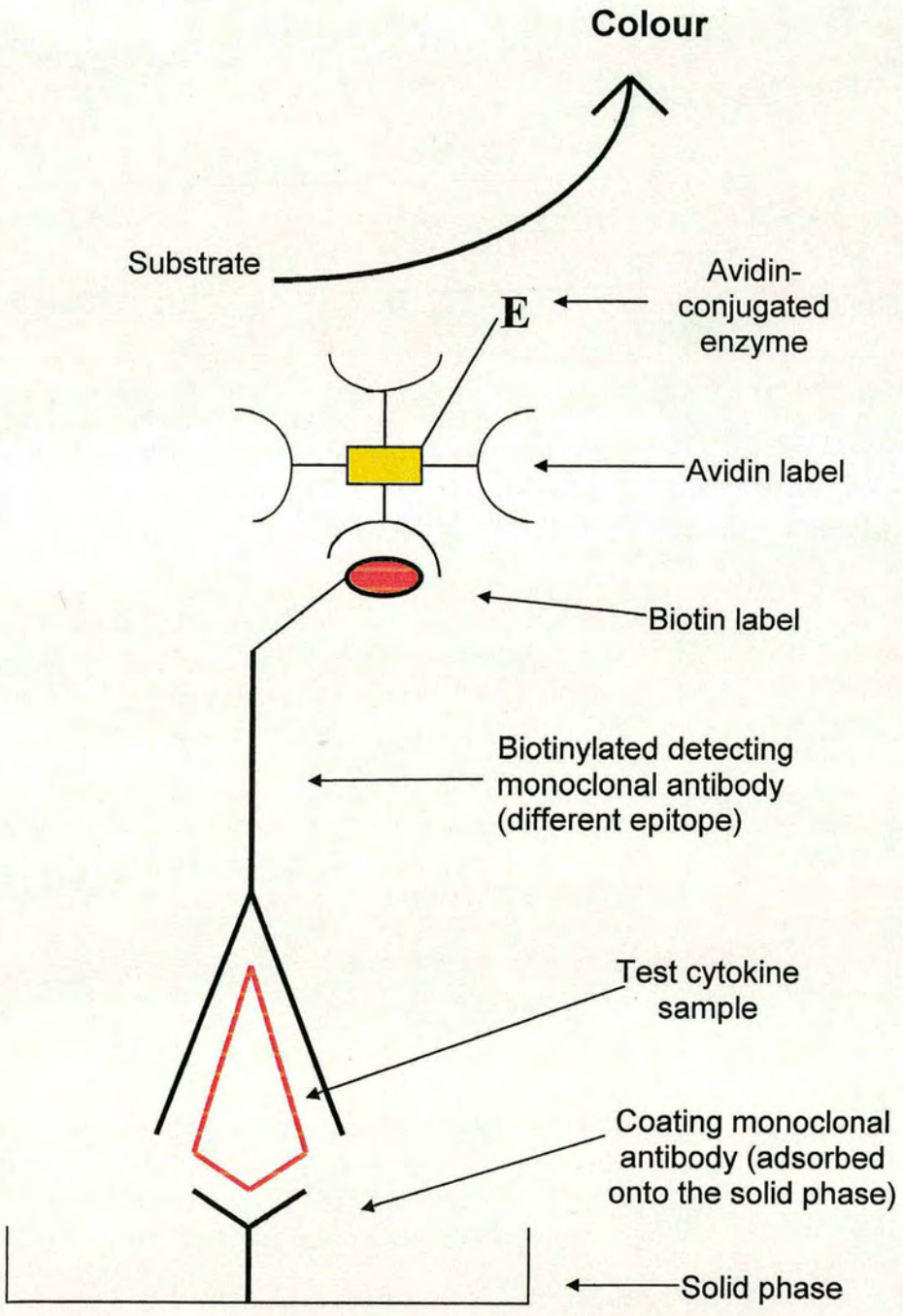


Table 3.1: Complete protocol for all cytokine ELISAs (for the list of all reagents, refer to section 2.6.1).

1. Add 100 µl/well of capture Ab at the appropriate dilution in coating buffer.
2. Incubate plates overnight at RT.
3. Wash plates 4 times with 300 µl/well of wash solution.
4. Add 200 µl/well of blocking buffer.
5. Incubate plates for 1 hour at 37°C.
6. Wash plates 3 times with 300 µl/well of wash solution.
7. Add 50 µl/well of standard or culture supernatant.
8. Incubate plates for 3 hours at RT.
9. Wash plates 4 times with 300 µl/well of wash solution.
10. Add 50 µl/well of biotinylated detecting Ab at the appropriate dilution in blocking buffer.*
11. Incubate plates for 1 hour at RT.
12. Wash plates 6 times with 300 µl/well of wash solution.
13. Add 100 µl/well of avidin-labeled horseradish peroxidase at the appropriate dilution in blocking buffer.
14. Incubate plates for 30 minutes at RT.
15. Wash plates 8 times with 300 µl/well of wash solution.
16. Add 100 µl/well of OPD substrate.
17. Incubate plates for 15 minutes at RT.
18. Add 25 µl/well of stop solution.
19. Read absorbance at 492 nm.

*Detecting Ab for IL-12 p40 was added at 100 µl/well.

Table 3.2: Reagent concentrations used for each cytokine ELISA (all reagents are from TCS unless otherwise indicated).*

	IFN-γ	IL-12 p70[!]	IL-12 p40[#]	IL-10
Coating Ab	2	2	4	2
Detecting Ab	0.05	0.25	0.3	0.05
HRP	0.5	0.5	1	1

*All values are given in μg/ml.

[!] Recombinant IL-12 p70 standard from PeproTech EC Ltd (London, UK).

[#] IL-12 p40 reagents are all from R&D Systems (Abingdon, UK).

Figure 3.2: Standard curves for a) IFN-γ, b) IL-12 p70, c) IL-10, and d) IL-12 p40 comparing varying concentrations of coating Ab with optimal concentrations of detecting Ab for each cytokine. 10 (●), 5 (■), 2 (▲) and 1 (◆) μg/ml for IFN-γ, IL-12 p70 and IL-10. 4 (●), 2 (■) and 1 (▲) μg/ml for IL-12 p40.

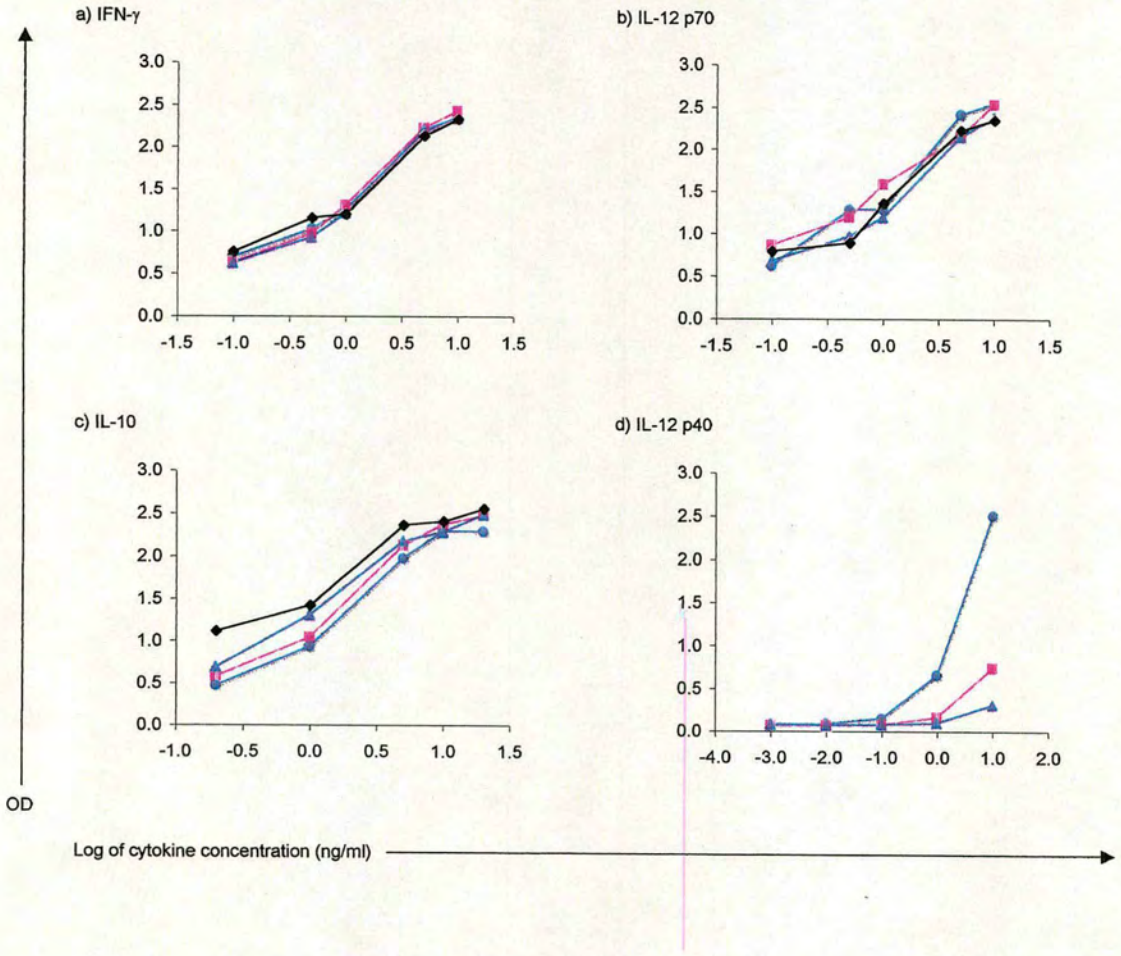


Figure 3.3: Standard curves for a) IFN-γ, b) IL-12 p70, c) IL-10, and d) IL-12 p40 comparing varying concentrations of detecting Ab with optimal concentrations of coating Ab for each cytokine. 1 (●), 0.25 (■), 0.05 (▲) μg/ml for IFN-γ, IL-12 p70 and IL-10. 0.3 (●), 0.15 (■) and 0.075 (▲) μg/ml for IL-12 p40.

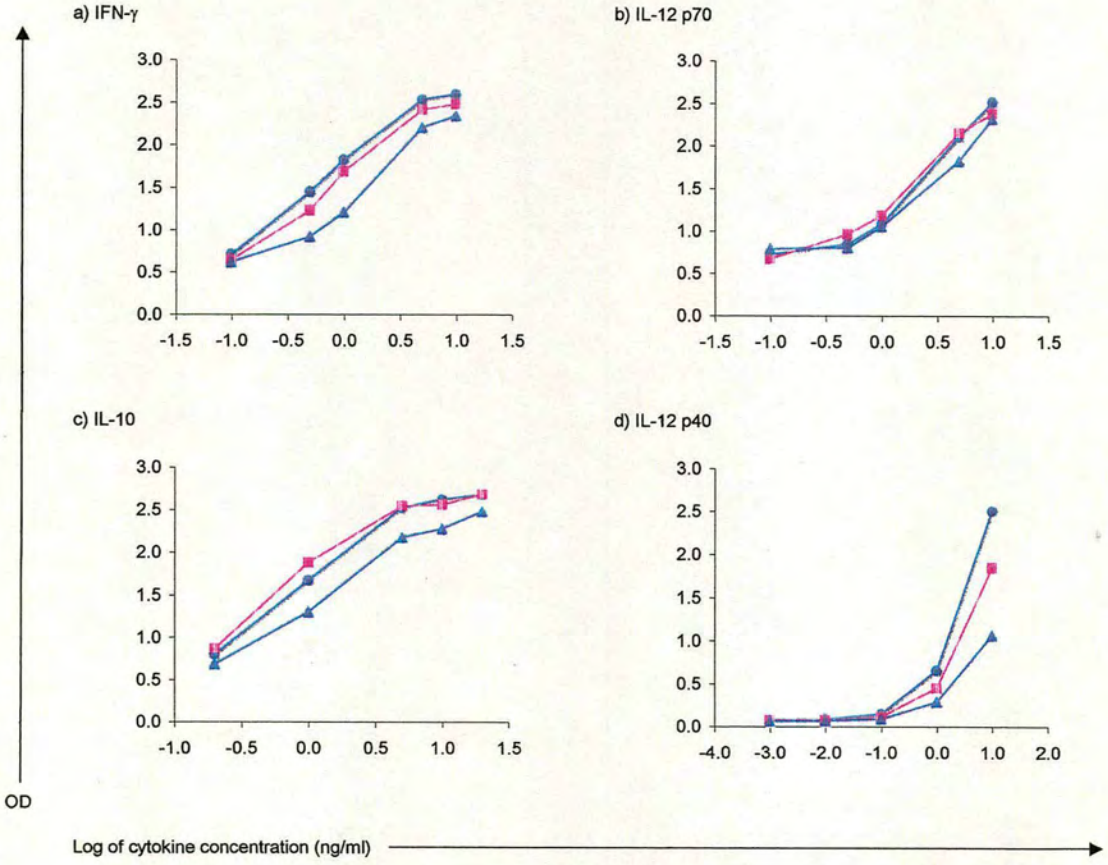


Figure 3.4: Standard curves for a) IFN-γ, b) IL-12 p70, c) IL-10, and d) IL-12 p40 comparing 50 (●) versus 100 (■) µl of standard.

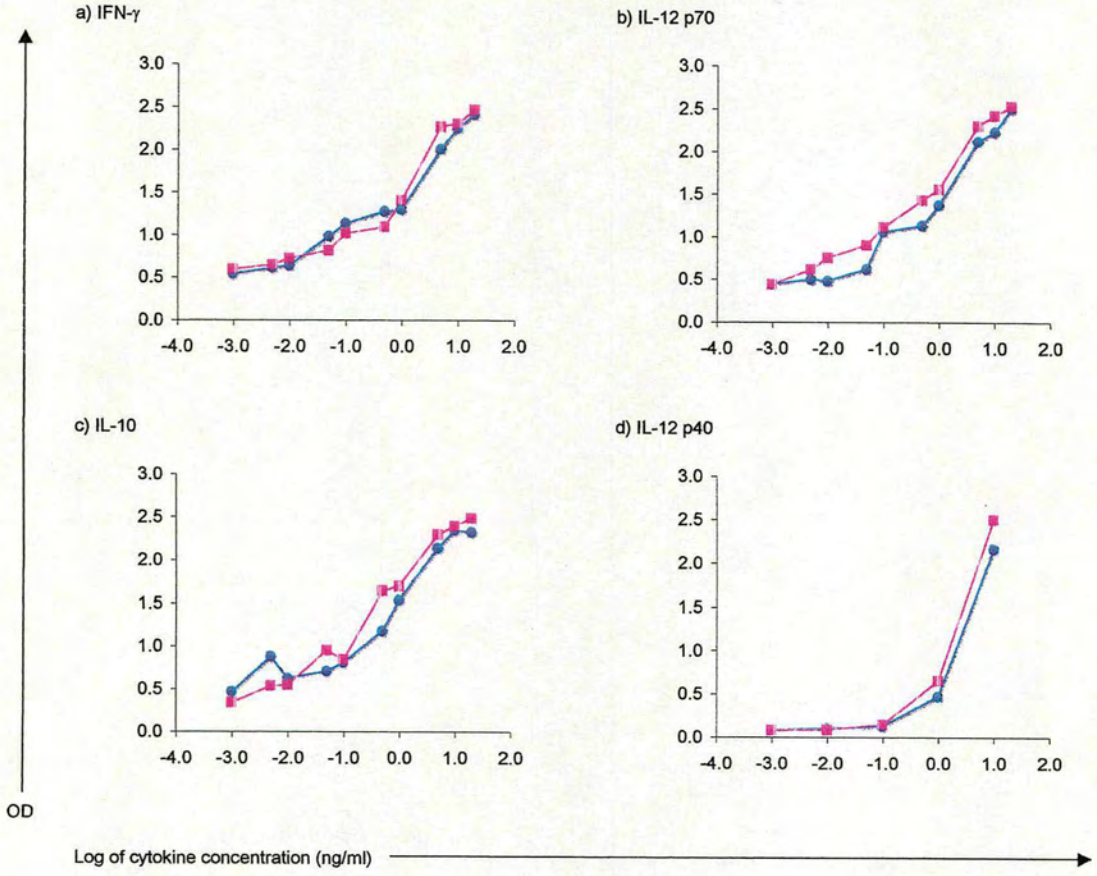


Figure 3.5: Standard curves for a) IFN-γ, b) IL-12 p70, c) IL-10, and d) IL-12 p40 comparing 2.5 (●), 1 (■) and 0.5 (▲) μg/ml of HRP.

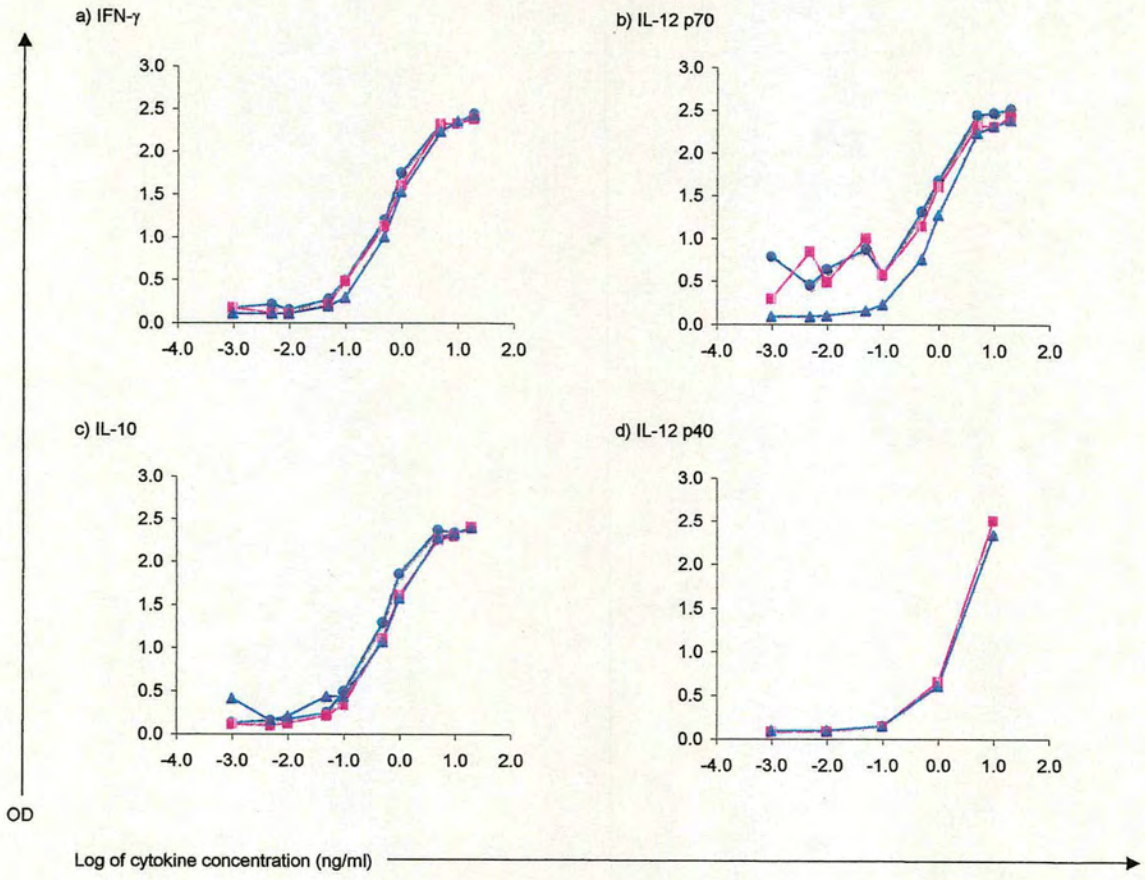


Figure 3.6: Standard curves for a) IFN-γ, b) IL-12 p70 and c) IL-10 comparing 37°C (●) versus RT (■) for the blocking step (IL-12 p40 ELISA was not assayed).

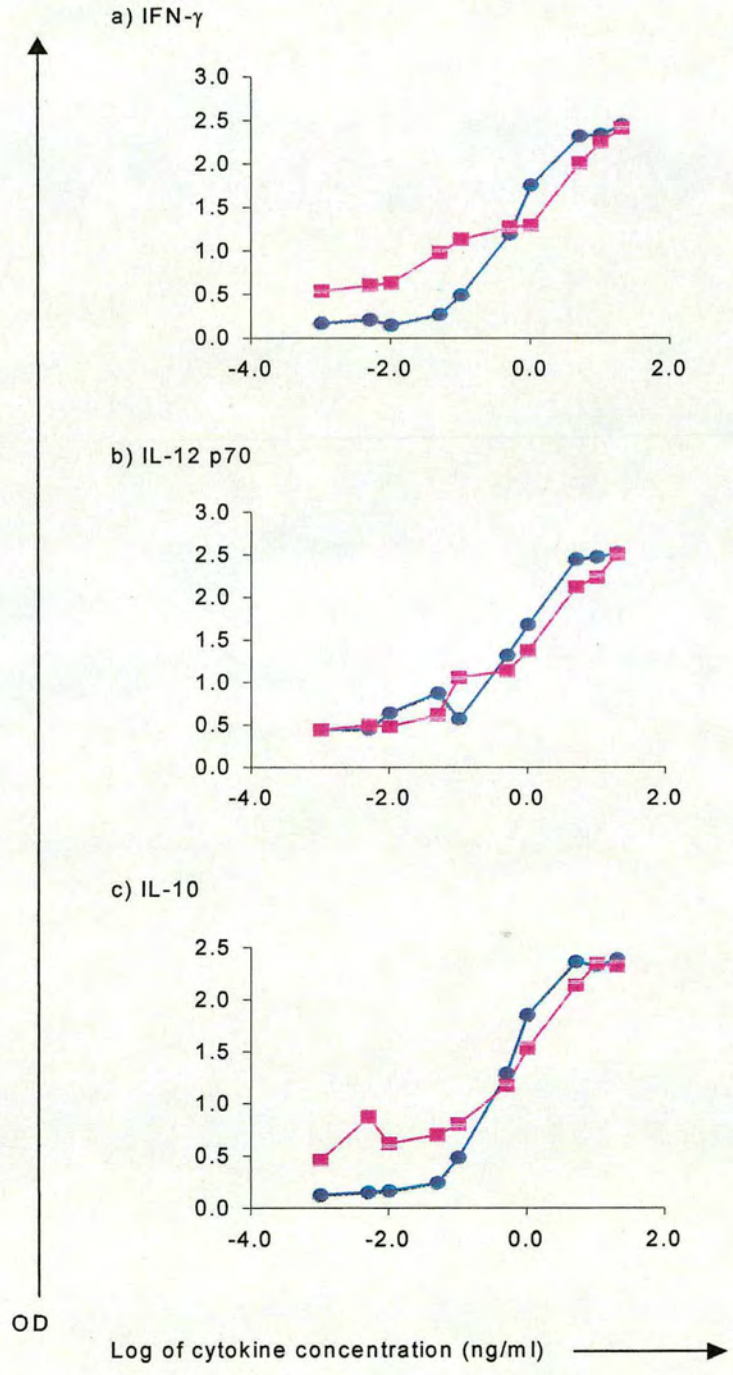


Figure 3.7: Standard curves for a) IFN-γ, b) IL-12 p70 and c) IL-10 comparing 4% BSA (●) versus 1% skimmed milk (■) as diluent and blocking buffers (IL-12 p40 ELISA not assayed).

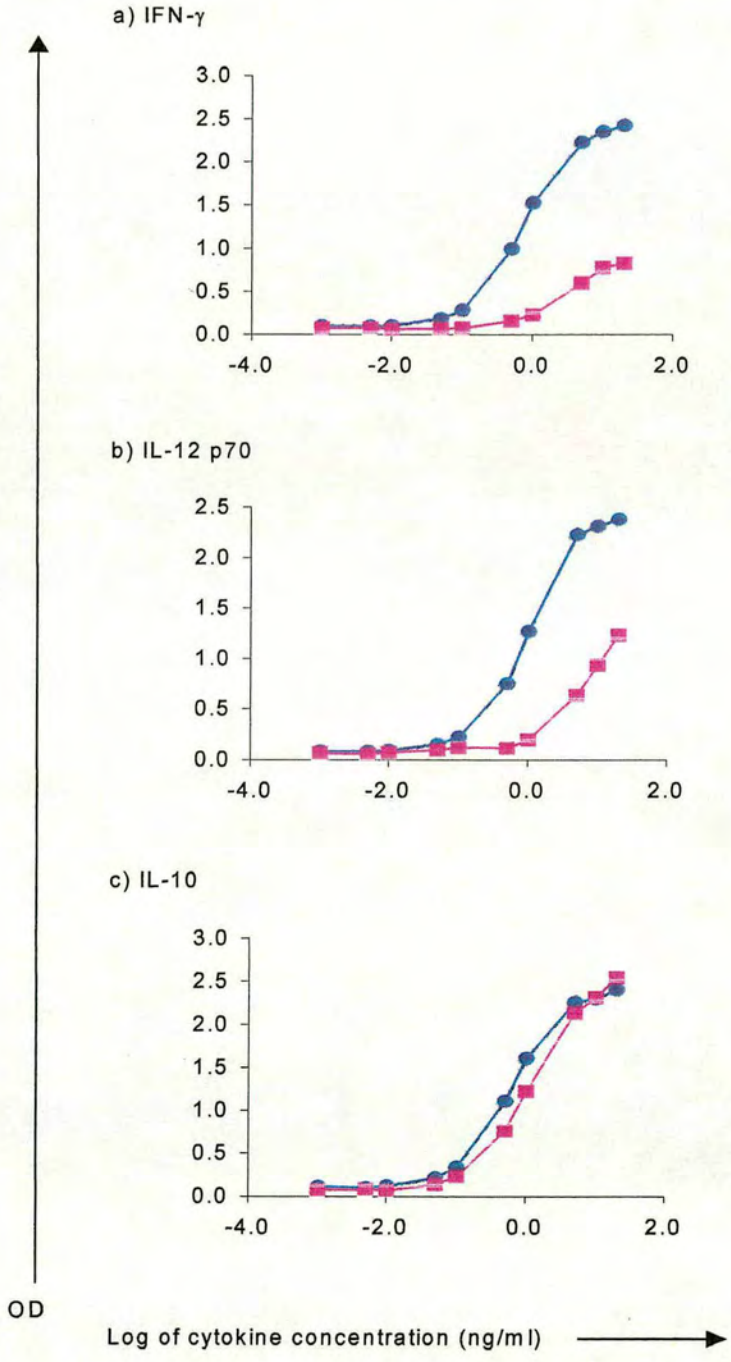


Figure 3.8: Given (●) versus predicted (■) concentrations of a) IFN-γ, b) IL-12 p70, c) IL-10, and d) IL-12 p40. Linear regression analysis was done on the points which gave the best fit curve.

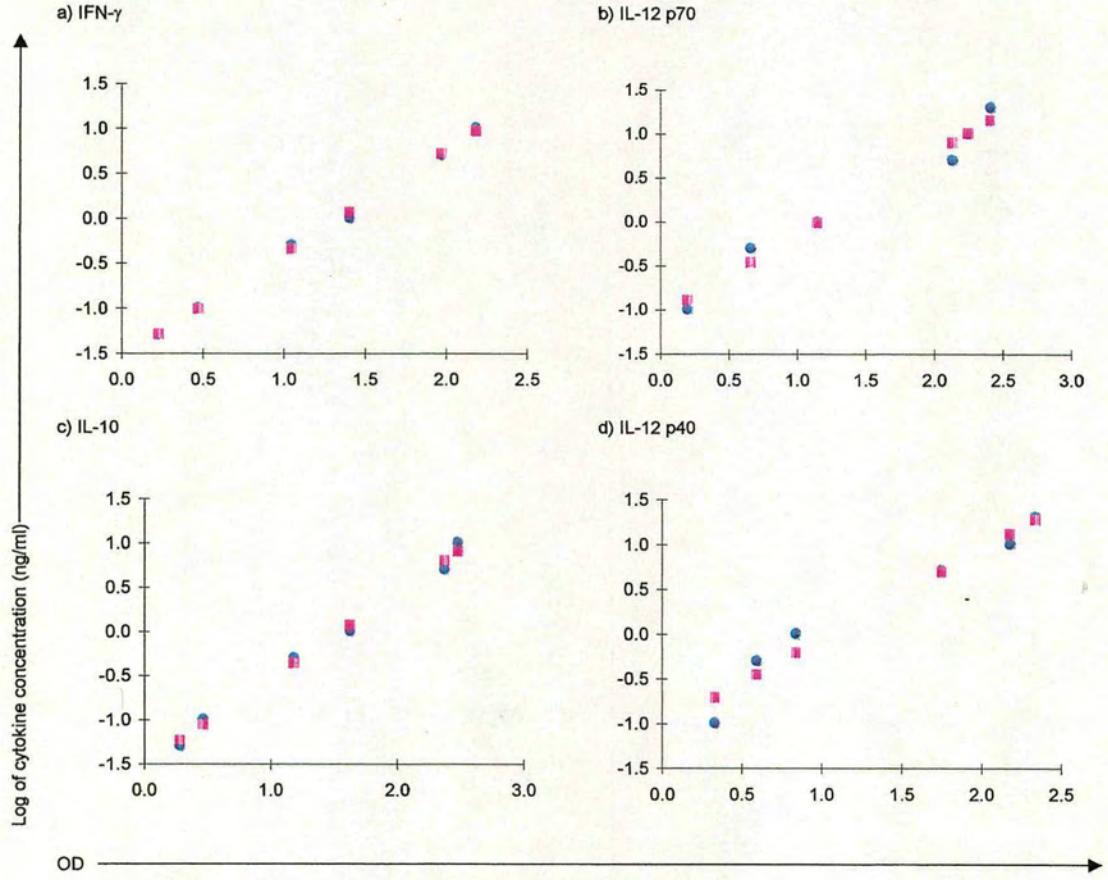
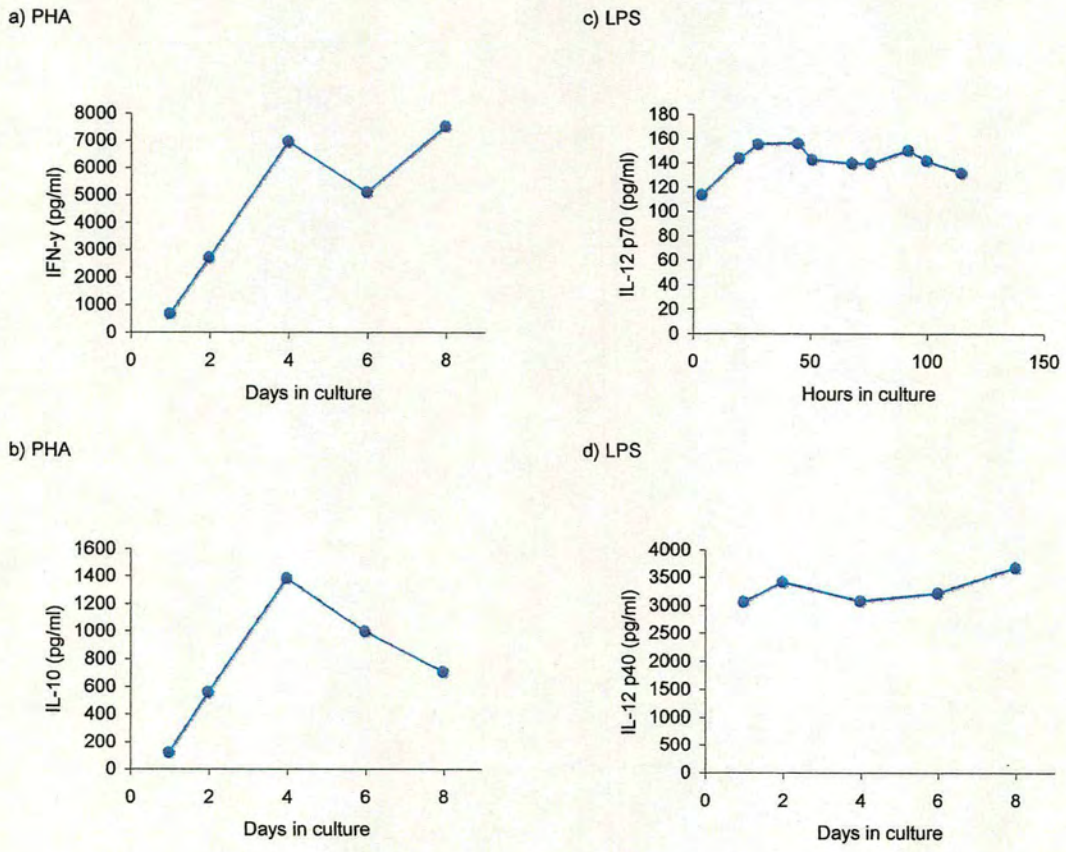


Figure 3.9: Representative production of a) IFN-γ and b) IL-10 from PHA-stimulated blasts and c) IL-12 p70 and d) IL-12 p40 from LPS-stimulated PBMCs.



Chapter 4: Optimisation of reverse transcriptase polymerase chain reaction (RT-PCR) for IL-12 messenger RNA (mRNA) detection

4.1 Introduction

RT-PCR, a variation of PCR, is one of the most useful tools for studying gene regulation and expression. In short, messenger RNA is reverse transcribed to its complementary DNA by a reverse transcriptase. Specific DNA sequences are subsequently amplified by primer extension in PCR.

There are a number of molecular techniques, such as *in situ* hybridisation and Northern blot, which have been developed for measuring gene expression. But of these, RT-PCR is the most versatile. RT-PCR requires much less RNA, is less time consuming and more sensitive than the other assays.

RT-PCR has often been used to study cytokine expression during immune responses (30, 38, 111, 156, 204, 226, 241, 317). Although RT-PCR does not measure secreted cytokine and cytokine transcripts are often difficult to quantify, it is not always possible to use standard assays such as ELISA. Analysing cytokine mRNA levels is appropriate when there are too few cells (38), when a number of cytokines need to be measured at one time (317) and when measuring cytokines which rapidly get used up, have a short half-life or are produced at very low levels.

4.1.1 Principles of assay optimisation

4.1.1.1 RNA isolation

Choosing total RNA or poly (A)+ RNA isolation method depends on the downstream application. Although poly (A)+ RNA isolation highly enriches for mRNA, total RNA extraction methods (often based on guanidinium thiocyanate) may be preferred if there is a high throughput of samples (200, 273).

The most important aspect of RNA extraction is keeping an RNAse-free environment in order to prevent RNA degradation. Extraction of RNA should be done as quickly as possible, because RNAses contained in secretory vesicles are released when cell surfaces are disrupted (150). Using sterile disposable plasticware, handling samples with gloves, and diethyl pyrocarbonate (DEPC)-treating glassware are just some of the measures which should be followed when working with RNA (200, 273).

4.1.1.2 First strand synthesis

The choice of reverse transcriptase and cDNA primer are the 2 main aspects of first strand synthesis. The most commonly used reverse transcriptases are derived from Avian myeloblastosis virus and Moloney murine leukemia virus (202, 274). Random, oligo-(dT) and specific priming are the 3 ways to prime cDNA. Random priming is beneficial for detecting rare mRNA transcripts but is not often used (202). Oligo-(dT) is often the method of choice, because more than one transcript can be detected (65).

A one step-one tube system which speeds up RT-PCR and reduces risk of contamination has been developed (274). However multiple transcripts cannot be measured, and it has been reported that reverse transcriptase can inhibit Taq DNA polymerase (81).

4.1.1.3 PCR assay parameters

Primers are the single most important aspect which determines the success of PCR amplification. There is no one best way to design primers, but most range from 15-30 bp in length with 40-60% G-C content (272). Primers with complementary 3' ends, significant secondary structure, runs of 3 or more G's and C's at 3'ends, and significantly different melting temperatures should be avoided (148, 272, 296).

Concentration and pH are important parameters for the PCR master mix. Concentrations of $MgCl_2$, dNTP and primers which are too high reduce the specificity of the reaction (148, 172, 272, 296, 329). However, molar excess of reagents compared to template drives the reaction (172). Buffer pH must not be too high as it decreases the fidelity of the Taq or too low as it damages DNA (80).

The choice of DNA polymerase is also important. As errors in sequencing occur, using T4 or T7 DNA polymerases which have proof-reading ability is beneficial. Because these polymerases are not stable at high temperatures, Taq DNA polymerase is more often used, (80). As non-specific priming occurs even when the reaction mix is sitting at RT, it is also recommended to use a 'hot-start' polymerase which is only activated at denaturing temperatures (7).

Finally cycling parameters need to be optimised. High annealing temperatures increase the specificity of the reaction (148, 172, 272, 296, 329). Annealing

temperatures ranging between 55-60°C is usually recommended. Amplification reactions should proceed for, at most, 40 cycles. The reaction reaches a plateau phase where the efficiency is decreased and nonspecific priming occurs when the number of cycles is too high (172, 297).

4.1.2 Quantitative methods of RT-PCR

PCR products are normally separated on EtBr-stained agarose gels and visualised under ultraviolet light, although fluorescent labeled probes are often used because they afford greater sensitivity (10). These methods are only qualitative, but in some cases, such as when cytokines are up-regulated, a quantitative assay is necessary.

Non-competitive methods have been used for a variety of purposes (30, 140, 201, 226) and are divided into 2 basic categories: titration and kinetic. Although non-competitive RT-PCR is fairly easy to optimise and can use external controls, it does not control for variations in tube-to-tube amplification efficiency and can only tell how much product there is relative to other samples.

Competitive methods where internal controls are co-amplified with DNA targets are more often used. Endogenous RNA such as β-actin can be used (178, 241) as an internal control, but exogenous RNA is more often used as a competitor molecule. A number of studies use synthetic RNA competitors which are amplified by the same primers but differ in length with the target sequence (36, 111, 204, 327, 352, 353). However, it is not known whether the amplification efficiency of the standard is equal to the target. Other studies avoid this complication by using a competitor molecule having an almost identical sequence except for a single point mutation of the target sequence (19, 131, 162). Although these methods are better for absolute quantitation than assays using external or endogenous RNA controls, developing synthetic controls is relatively time-consuming.

4.2 Optimisation of RT-PCR for measuring IL-12 p40 responses to *P. falciparum* antigen *in vitro*

The complete RT-PCR assay is summarised in section 2.7.

4.2.1 Total RNA isolation

All RNA preparations were done using a commercial RNA extraction kit (RNeasy mini kit with Qiashredder; Qiagen; Crawley, UK) or with RNAzol B (Biogenesis; Poole, UK). Both methods worked equally well for preparing total RNA for RT-PCR assays (figure 4.1). Total RNA extraction methods were chosen over poly(A)+ RNA methods primarily because of the cost, the relative ease of the assay and the large number of samples to be processed. Briefly, 10^6 cells were pelleted, lysed and homogenised with 1 ml of RNAzol B containing guanidinium isothiocyanate. One hundred microliters of chloroform (Sigma) were added to the cell suspension, shaken for 15 seconds, and incubated at 4°C for 5 minutes. The suspension was centrifuged for 15 minutes at 12,000 g at 4°C to extract the RNA into the aqueous phase. The soluble RNA was then transferred to a fresh tube and isopropanol (Sigma) was added at a 1:1 volume and incubated at 4°C for 15 minutes. RNA was precipitated by centrifuging for 15 minutes at 12,000 g at 4°C. The pellet was washed in 75% ethanol (Hayman Limited; Essex, UK), centrifuged for 8 minutes at 7,500 g at 4°C, and resuspended in 50 µl of RNAse-free water. The amount of RNA in the sample was not quantified with a spectrophotometer at this time, because there was very little starting material. RNA sample-to-sample variation was controlled for by visually equalising (under ultraviolet light) the housekeeping transcript, β-actin.

In order to remove any contaminating genomic DNA, RNAse-free DNAse (Boehringer-Mannheim) was added in excess of 1 U/µg RNA and then incubated at 37°C for 30 minutes, 75°C for 5 minutes. Samples were either used immediately for first strand synthesis or stored in 100% ethanol at –70°C until required.

4.2.2 First strand synthesis

cDNA was reverse transcribed from 11 µl (or less) RNA in a final volume of 20 µl with 1 µl (0.5 µg) oligo $(dT)_{12\text{-}18}$ primer (Gibco Life Technologies) as the primer. Oligo-$(dT)_{12\text{-}18}$ primer was chosen because more than one transcript was being measured. Total reaction solution also included the following:

1. 2 µl 0.1M dithiothreitol (DTT; Gibco Life Technologies),
2. 4 µl 5X first strand buffer (250 mM Tris pH8.3, 375 mM KCl, 15 mM $MgCl_2$; Gibco Life Technologies)

3. 1 µl (10 mM) dNTP mix

4. 1 µl (200 U) superscript II reverse transcriptase (RT; Gibco Life Technologies).

Synthesis was run at 42 °C for 50 minutes and then stopped by raising the temperature to 70 °C for 15 minutes.

4.2.3 PCR

4.2.3.1 PCR controls

The following controls were run for each PCR assay:

1. β-actin, a constitutively expressed gene, was used as a positive control to show evidence of RNA in every sample, to control for variability between samples and to confirm the reverse transcription and PCR reactions.

2. Plasmids containing both β-actin and IL-12 p40 sequences were used as positive controls in order to make sure the PCR reaction worked (see section 4.2.4).

3. A sample containing no RNA was used as a negative control for contamination in the RT-PCR reagents.

4. An RNA sample with no RT step was used for a negative control for genomic DNA contamination.

4.2.3.2 Primers

Oligonucleotide sequences for IL-12 p40 primers were sourced from published sequences (364) and β-actin primers were designed from the GEN-EMBL database. Primers were designed to sit on each side of at least one intron in order to distinguish between genomic and cDNA fragments, except for IL-12 p40 primers for which the genomic DNA had not been sequenced. IL-12 p40 primers were tested with human genomic DNA (Boehringer-Mannheim), and no 374 bp band was detected, although a band >2000 bp was detected (data not shown). β-actin and IL-12 p40 primers were also designed to work at the same efficiency (same melting point and equivalent numbers of base pairs with 55% GC content) in order to use them in semi-quantitative analysis (see section 4.2.5). Sequence information is shown in table 4.1.

4.2.3.3 PCR assay parameters

Concentrations of $MgCl_2$ and Taq were optimised (figures 4.2-4.3). Promega Taq DNA polymerase and its corresponding buffer (see section 2.1.2 for details) were chosen for thermostability and cost. Variations in these parameters did not affect β-actin transcripts but were important in optimising conditions for IL-12 p40 transcripts. With low concentrations of $MgCl_2$ and high concentrations of Taq, no IL-12 p40 transcripts were detected. Variations in primer and dNTP concentrations did not affect PCR reactions for either transcript (data not shown). Equal concentrations of dATP, dCTP, dGTP, and dTTP were used. The optimal PCR mix thus contained the following:

1. 1 μl cDNA
2. 1 μl (1μM) each cytokine primer (Oswel, Southampton, UK),
3. 0.8 μl (2 mM) $MgCl_2$
4. 0.2 μl (200 μM) dNTP mix
5. 1 μl 10X buffer
6. 0.1 μl (0.5 U) Taq DNA polymerase
7. Volume made up to 10 μl with sterile distilled water.

PCR amplification was performed for 35 cycles for β-actin and 35 or 40 cycles for IL-12 p40, because of the relative rarity of IL-12 p40 starting material. Cycle numbers were not increased to more than 40 cycles, because there was greater risk of nonspecific priming. Annealing temperature was also optimised. At lower temperatures, there was a lot of nonspecific binding for IL-12 (figure 4.4). The temperatures used for PCR were: denature 94°C for 1 minute, primer annealing 60°C for 1 minute, extension 72°C for 1 minute. Assay-to-assay variation was minimal as the same Hybaid thermocycler was used for each assay (figure 4.5).

4.2.3.4 Analysis

PCR products were analysed by gel electrophoresis. PCR fragments were separated on EtBr-stained, 1.5% agarose gels run in 0.5X TBE buffer (5.4 g trizma base, 2.75 g boric acid, both from Sigma; 2 ml 0.5 M EDTA pH 8, Boehringer-Mannheim, in 1 L distilled water). Forty percent of each sample was run in 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, both from Sigma; 15% ficoll type 400, Amersham-Pharmacia) with 100 base pair DNA molecular weight marker (3 μl

of 125 mg/ml solution; Boehringer-Mannheim) to determine size of PCR products. PCR products were visualised under ultraviolet light and analysed with Multi Analyst software package (Biorad, Hertfordshire, UK).

4.2.4 Fidelity of PCR product by sequencing

4.2.4.1 Solutions used in plasmid preparation

1. LB broth pH 7
   10 g sodium chloride (Sigma)
   10 g bacto-tryptone (Boehringer-Mannheim)
   5 g bacto-yeast extract (Boehringer-Mannheim)
   1 L deionised water
2. LB ampicillin agar plates pH7
   LB broth
   20 g bacto-agar (Boehringer-Mannheim)
   50 mg ampicillin (Sigma)

4.2.4.2 Preparation of plasmid control

β-actin and IL-12 p40 DNA were purified from PCR products using a commercial purification kit (High Pure PCR Product Purification Kit; Boehringer-Mannheim). DNA was resuspended in 10 µl of water and 7 µl of each suspension was used to clone DNA into plasmids using a commercial kit (pGEM®-T Easy Vector System; Promega). After the ligation reactions were run overnight, ligates were added to competent cells (XL1-blue, a strain of *Escherichia coli*; Stratagene; Amsterdam, Netherlands) and incubated for 1 hour on ice. The cell suspensions were heat shocked for 45 seconds at 42°C, incubated on ice again for 15 minutes, and then added to 300 µl of LB broth to incubate at 37°C for at least 1 hour. Two hundred microliters of cell suspension were plated onto LB-Ampicillin plates containing 0.1 M isopropyl-B-D-thiogalactopyranoside (IPTG) and 50 ng/ml 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-GAL)(both from Boehringer-Mannheim). Plates were incubated at 37°C overnight and transformed colonies (white colonies) were selected either for β-actin and IL-12 p40 PCR assays or for sequencing reactions to test the fidelity of amplified PCR products (see section 4.2.4.3).

Plasmids were purified with a commercial kit (Qiaprep Spin Miniprep Kit, Qiagen), and restriction digests were performed overnight at 37°C using 41 µl of plasmid, 5 µl of buffer 4 (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM DTT, pH7.9; New England Biolabs; Hitchin, UK) and 100 U of NDE1 or 200 U NSI1 restriction enzyme (both from New England Biolabs). The plasmid containing β-actin was then treated with alkaline phosphatase (so that it would not religate onto itself) in a reaction containing 10 µl plasmid, 2 µl buffer (50 mM Tris-HCl pH 8; Amersham), 2 U shrimp alkaline phosphatase (Amersham), and 6 µl of water. The reaction was run at 37°C for 30 minutes and then stopped by raising the temperature to 80°C for 10 minutes. Plasmids containing β-actin or IL-12 p40 were then ligated using the method described above.

4.2.4.3 Sequencing β-actin and IL-12 p40

Fidelity of the amplified PCR product was confirmed by using a commercial sequencing kit (ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit; Perkin Elmer-Applied Biosystems; Warrington, UK) using universal vector primers. After the purified plasmid was sequenced and run on a gel, the PCR product was purified by ethanol precipitation. Briefly, 2 µl of 3 M sodium acetate pH 5.5 (BDH), 50 µl 100% ethanol and 20 µl of the reaction product were incubated at -20°C for 30 minutes and then centrifuged for 20 minutes at 12,000 g at 4°C. The DNA pellet was washed once in 70% ethanol and resuspended in 3 µl of loading buffer (5:1 ratio of deionised formamide, BDH to 25 mM EDTA pH 8 containing 50 mg/ml blue dextran, BDH). The reaction mixture was denatured at 90°C for 2 minutes and then run overnight on an automated sequencer (Perkin Elmer-Applied Biosystems). The sequences were analysed with ABI PRISM™ 377 Gene Scan Analysis software and confirmed to be β-actin or IL-12 p40 by comparing sequences to the GEN-EMBL database.

4.2.5 Semi-quantitative RT-PCR optimisation

IL-12 was originally measured by IL-12 p70 ELISA, but no significant IL-12 was detected from PfSE-stimulated PBMCs (see chapter 6). In order to determine amounts of IL-12 being produced, optimisation of a non-competitive, semi-quantitative RT-PCR was then begun. Relative levels of IL-12 p40 would be determined by co-amplification

with β-actin. A plasmid containing both β-actin and IL-12 was developed to demonstrate that primers for these transcripts worked at equal efficiency (figure 4.6). However, subsequently semi-quantitative RT-PCR was discontinued, because qualitative RT-PCR assay turned out not to be as sensitive as the IL-12 p40 ELISA (see section 4.3).

4.3 Results

4.3.1 Study population and cell cultures

Venous blood was taken from 13 naïve individuals, 20 exposed, but not clinically immune, individuals and 3 control individuals and processed for *in vitro* PBMC culture (study populations and method of cell separation are described in Chapter 2). PBMCs were distributed at 10^6/well in a total of 1 ml of complete culture medium in flat-bottomed 24-well plates and cultured for 1, 2, 4, 6, and 8 days with uRBC or PfSE. PHA or LPS were used as positive control stimuli for cytokine production. Adherent and non-adherent PBMCs were harvested and pelleted for RNA extraction and subsequent RT-PCR as described in section 4.2.

4.3.2 RT-PCR does not show high levels of IL-12 transcripts

Figure 4.7 shows representative gels for the complete assay of 1 exposed donor, E10. All positive and negative control PCRs worked for donor E10. β-actin PCRs were all positive except for PBMCs stimulated with uRBC on day 6. However, there were no IL-12 transcripts detected even for the LPS-stimulated cells. PfSE-stimulated IL-12 p40 transcripts with β-actin controls for cells of all individuals are shown in figures 4.8-9 (control individuals not shown). Positive plasmid and negative (no DNA) controls worked consistently for all individuals. RNA was demonstrated for almost all samples by β-actin transcripts, and very few samples had genomic DNA contamination. The PCR reaction sometimes primed up β-actin genomic DNA transcripts, but even in those samples, cDNA transcripts were dominant (complete details for each individual are given in Appendix 1, tables A1.1-A1.3).

For both naïve and exposed individuals, PfSE-stimulated IL-12 p40 was detected more often on day 1 than any other day. On day 1, 38% and 20% had positive responses in naïve and exposed populations (5/13 and 4/20 positive responders

respectively; table 4.2). However, overall naïve individuals had more positive IL-12 responses to PfSE than exposed (77%, 10/13 and 30%, 6/20 respectively). uRBC also stimulated IL-12 p40 production almost as much as PfSE in cells from naïve donors and equally as much in PBMCs from exposed donors on day 1. Although uRBC has been shown to non-specifically stimulate PBMCs (see Chapter 7), it is surprising that almost equal numbers of exposed individuals produced IL-12 p40 to uRBC and PfSE (naïve individuals had higher cumulative responses to PfSE by day 6). Conversely, IL-12 p40 was not detected in all of the positive (LPS/PHA) controls (91%, 10/11 and 35%, 7/20 cumulative responses in naïve and exposed donors respectively). Also, more naïve donors produced IL-12 p40 to PfSE in comparison to exposed donors (table 4.2). This result was surprising in light of the fact that exposed individuals produced more IFN-γ in response to PfSE than naïve individuals and that a significant proportion of that IFN-γ was IL-12 dependent (see Chapter 6).

Because the data seemed unusual and because there was not enough IL-12 p40 transcript produced to quantify, an IL-12 p40 ELISA was used to confirm the RT-PCR results (analysis of IL-12 p40 ELISA results is found in Chapter 6). IL-12 p40 protein was measured on day 2 and compared with cumulative transcript responses to uRBC or PfSE (tables 4.3-4.4). IL-12 p40 protein levels were considered positive if they were above the LLD (171pg/ml). IL-12 p40 ELISA was more sensitive than RT-PCR for both groups overall (table 4.5). ELISA detected significantly more IL-12 p40 responders to uRBC and PfSE in the exposed group (Chi-squared, df=1, χ^2=23.02, p<0.001 for uRBC and χ^2=12.38, p<0.001 for PfSE). All the individuals who had positive IL-12 responses detected by RT-PCR were also positive by ELISA, except for donors N16 and N2 (in response to uRBC and PfSE respectively). Although different numbers of responders were detected by each assay, ELISA confirmed RT-PCR analysis in that there were almost an equivalent number of responders to uRBC and PfSE.

4.4 Discussion: Is RT-PCR the best way to measure IL-12?

4.4.1 Justification for using RT-PCR

IL-12 is an important cytokine in the inflammatory response to *P. falciparum*. It was demonstrated that IFN-γ production from PfSE-stimulated PBMCs of naïve,

exposed and immune individuals was downregulated when IL-12 was neutralised (see Chapter 6). I therefore wanted to directly measure IL-12 responses in the system.

IL-12 p70 ELISA was first used, but most samples tested had PfSE-stimulated IL-12 p70 levels below LLD (see Chapter 6). Although there is a standard bioassay for IL-12 p70 (109) that has been used to detect bioactive IL-12 (184), this assay was not chosen for a number of reasons. First, bioassays may not accurately reflect how much IL-12 is in the system, because IL-12 p40 homodimer can antagonise IL-12 p70 bioactivity (124, 133, 359). Second, bioassays are fairly complex to standardise, and results may be variable. Heufler *et al.* reported variable readouts with IL-12 p70 bioassay (133). Because it was difficult to measure detectable protein levels of IL-12 and because such small amounts of IL-12 are needed for bioactivity, RT-PCR was chosen for its sensitivity in detecting minuscule amounts of transcript.

4.4.2 Why was RT-PCR not as sensitive as ELISA?

It was demonstrated that ELISA was actually more sensitive in detecting IL-12 p40 than RT-PCR. There are a number of reasons which could possibly explain why RT-PCR might not have been as sensitive. First, there may have been problems with the optimisation of the assay itself. It is recommended to increase the number of cycles for rare transcripts. In this case, IL-12 PCR amplification was already increased to 40 cycles, the upper limit of cycle number without increasing chances of mispriming. Alternatively, template could have been degraded or impure. It has been shown that impurities such as haemoglobin and heparin (both of which can be found in this assay system) can inhibit the efficiency of PCR amplification (207). However, after separating PBMCs from whole blood, levels of heparin or haemoglobin should have been much lower than the concentrations which would effect PCR. Any number of variables could also have effected efficiency and sensitivity such as missing or poor quality reagents, suboptimal reaction conditions, inconsistent cycling, etc. All these variables were accounted for as stated in the results section, and for the most part, there was little assay-to-assay variation. Technical problems with the assay therefore seem to be an unlikely explanation.

It has been established that PfSE-stimulated PBMCs produce IL-12. Thus, the most likely reasons that IL-12 p40 could not be detected are because there was either

too little transcript or transcript production was too transient. The timepoints chosen may have been too late to detect IL-12 p40. Phagocytic cells show increased accumulation of IL-12 p40 transcripts 2-4 hours after being stimulated with LPS but subsequently decrease again after a few hours (212). Whole PBMCs may also not be the best population to detect IL-12 p40. It may have been better to separate out the possible IL-12 producing cells (macrophages or dendritic cells) in order to concentrate the specific RNA being measured.

There is no one assay that best measures IL-12. A number of studies have demonstrated that cytokines are detectable by one assay but not another (92, 357). In particular, Meyer zum Buschnfelde *et al.* demonstrated that changes in IL-12 levels from *Trypanosoma cruzi*-stimulated spleen cells could be detected by *in situ* hybridisation but not by semi-quantitative RT-PCR (231). Measuring mRNA may not be the best method for determining the relative importance of IL-12 in a system. There may be post-transcriptional factors such as inhibiting translation and protein degradation which regulate bioactive IL-12. For example, it has been demonstrated that TGF-β down-regulates TNF-α not through inhibiting transcription but through suppressing translation (34). Thus, depending on the aim of the study, a combination of different assays should be used to detect IL-12 expression.

However, the overall conclusion of both ELISA and RT-PCR methods is that parasite (PfSE-uRBC)-induced IL-12 cannot be consistently detected by either method, and the only clear demonstration of IL-12 activity was the assays when IL-12 was neutralised (see Chapter 6).

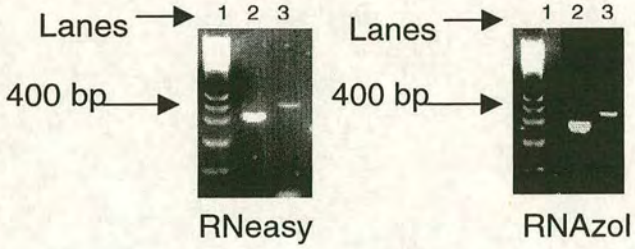
Table 4.1: Description of primers used for RT-PCR.

mRNA	Strand	Sequence	mRNA Location[!]	Fragment Size
β-actin	Sense*	CATGGGTCAGAAGGATTCCT	179	299
	Antisense*	AGAGGCGTACAGGGATAGCA	457	
IL-12	Sense*	GACCAGAGCAGTGAAGGTCTT	199	374
	Antisense*	CTCCTTGTTGTCCCCTCTGA	552	

* Sequences are read from 5' to 3'.

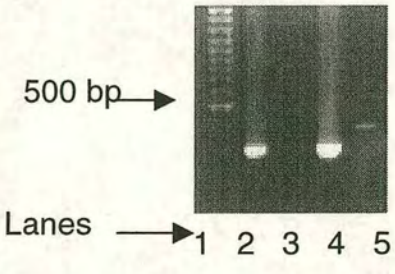
[!] Numbers indicate where the primer sequence begins.

Figure 4.1: Comparison of β-actin (299 bp) and IL-12 (374 bp) PCR products using different RNA extraction methods.



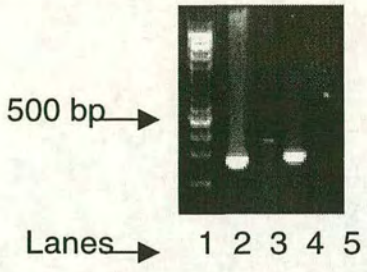
Lane 1= bp markers
Lane 2= β-actin
Lane 3= IL-12 p40

Figure 4.2: Comparison of β-actin (299 bp) and IL-12 (374 bp) PCR products using different of $MgCl_2$ concentrations in the PCR master mix (1.5 mM $MgCl_2$ for lanes 2 and 3, 2 mM $MgCl_2$ for lanes 4 and 5).



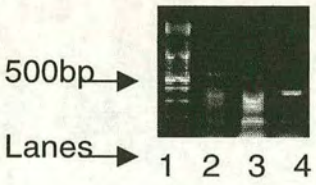
Lane 1= bp markers
Lane 2= β-actin
Lane 3= IL-12 p40
Lane 4= β-actin
Lane 5= IL-12 p40

Figure 4.3: Comparison of β-actin (299 bp) and IL-12 (374 bp) PCR products using different concentrations of Taq DNA polymerase concentrations in the PCR master mix (0.5 U Taq for lanes 2 and 3, 1 U Taq for lanes 4 and 5).



Lane 1= bp markers
Lane 2= β-actin
Lane 3= IL-12 p40
Lane 4= β-actin
Lane 5= IL-12 p40

Figure 4.4: Comparison of IL-12 (374 bp) PCR products using different annealing temperatures during PCR.



Lane 1= bp markers
Lane 2= 53 °C
Lane 3= 55 °C
Lane 4= 60 °C

Figure 4.5: Two-fold serial dilutions of β-actin (299 bp) and IL-12 (374 bp) plasmids for 6 different PCR assays demonstrating little assay-to-assay variation.

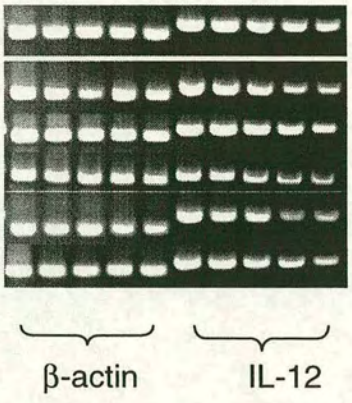


Figure 4.6: Plasmid containing both β-actin (299 bp) and IL-12 p40 (374 bp) transcripts, demonstrating that primers for these transcripts worked at equal efficiency.

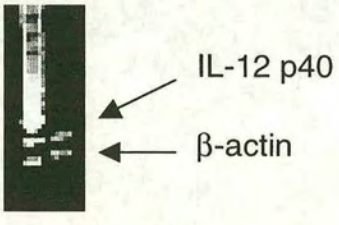
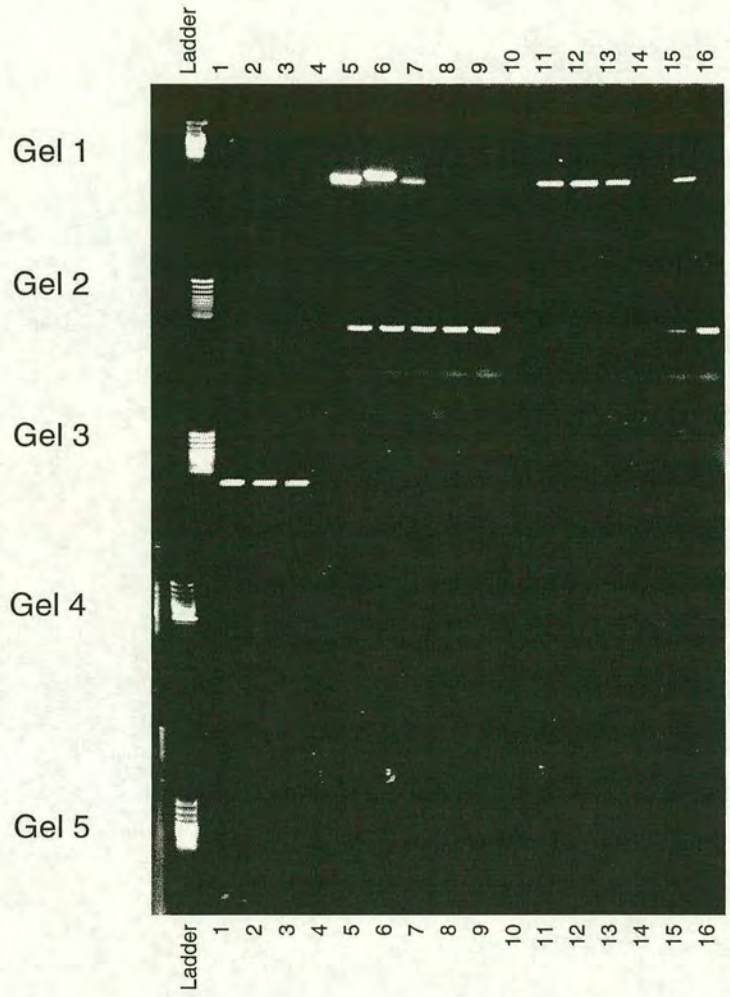


Figure 4.7: Complete RT-PCR assay (samples and controls) for donor E10 (gel key is given below).



Gel																			
	1			2			3			4			5		
Lane	Sample*	Primer		Sample	Primer		Sample	Primer		Sample	Primer		Sample	Primer	
1	no RNA	β-actin	-	uRBC2	IL-12	-	LPS4	β-actin	+	uRBC6/no RT	IL-12	-	LPS8/no RT	β-actin	-
2		IL-12	-	uRBC4	IL-12	-	LPS6	β-actin	+	uRBC8/no RT	IL-12	-	LPS1/no RT	IL-12	-
3	no cDNA	β-actin	-	uRBC6	IL-12	-	LPS8	β-actin	+	PfSE1/no RT	β-actin	-	LPS2/no RT	IL-12	-
4		IL-12	-	uRBC8	IL-12	-	LPS1	IL-12	-	PfSE2/no RT	β-actin	-	LPS4/no RT	IL-12	-
5	Plasmid	β-actin	+	PfSE1	β-actin	+	LPS2	IL-12	-	PfSE4/no RT	β-actin	-	LPS6/no RT	IL-12	-
6		IL-12	+	PfSE2	β-actin	+	LPS4	IL-12	-	PfSE6/no RT	β-actin	-	LPS8/no RT	IL-12	-
7	LPS#	β-actin	+	PfSE4	β-actin	+	LPS6	IL-12	-	PfSE8/no RT	β-actin	-			
8		IL-12	-	PfSE6	β-actin	+	LPS8	IL-12	-	PfSE1/no RT	IL-12	-			
9	LPS/no RT[!]	β-actin	-	PfSE8	β-actin	+	uRBC1/noRT	β-actin	-	PfSE2/no RT	IL-12	-			
10		IL-12	-	PfSE1	IL-12	-	uRBC2/no.RT	β-actin	-	PfSE4/no RT	IL-12	-			
11	uRBC1	β-actin	+	PfSE2	IL-12	-	uRBC4/no RT	β-actin	-	PfSE6/no RT	IL-12	-			
12	uRBC2	β-actin	+	PfSE4	IL-12	-	uRBC6/no RT	β-actin	-	PfSE8/no RT	IL-12	-			
13	uRBC4	β-actin	+	PfSE6	IL-12	-	uRBC8/no RT	β-actin	-	LPS1/no RT	β-actin	-			
14	uRBC6	β-actin	-	PfSE8	IL-12	-	uRBC1/noRT	IL-12	-	LPS2/no RT	β-actin	-			
15	uRBC8	β-actin	+	LPS1	β-actin	+	uRBC2/no.RT	IL-12	-	LPS4/no RT	β-actin	-			
16	uRBC1	IL-12	-	LPS2	β-actin	+	uRBC4/no RT	IL-12	-	LPS6/no RT	β-actin	-			

*Sample indicates which stimuli and day.
#LPS blasts from another donor.
[!]no RT indicates that there was no reverse transcription done (negative control for DNA contamination.
+' = band of correct size detected
-' = no band/ no band of correct size detected

Figure 4.8a: β-actin and IL-12 transcripts from PfSE-stimulated PBMCs for naïve individuals.

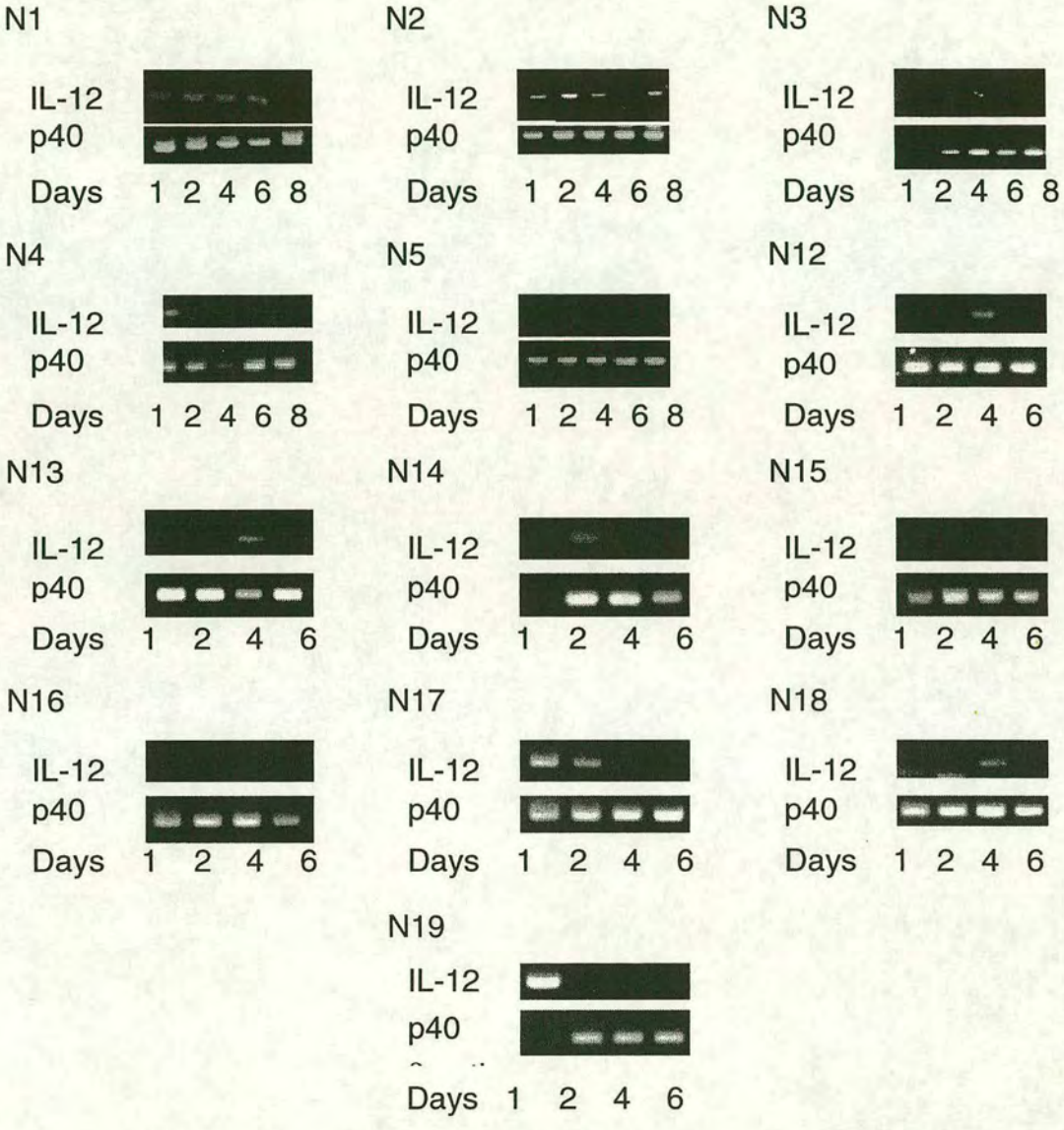


Figure 4.8b: β-actin and IL-12 transcripts from PfSE-stimulated PBMCs for exposed individuals.

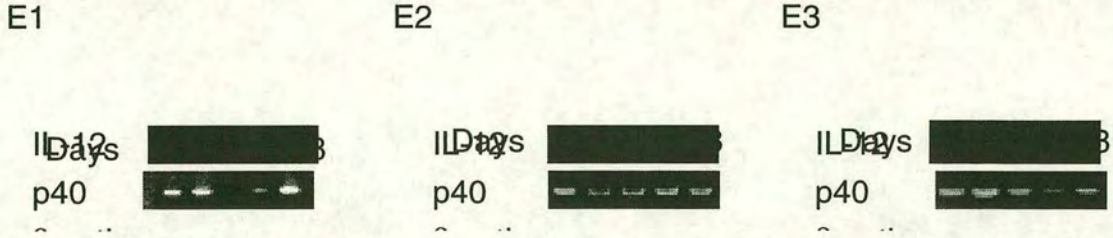


Figure 4.8b (continued):

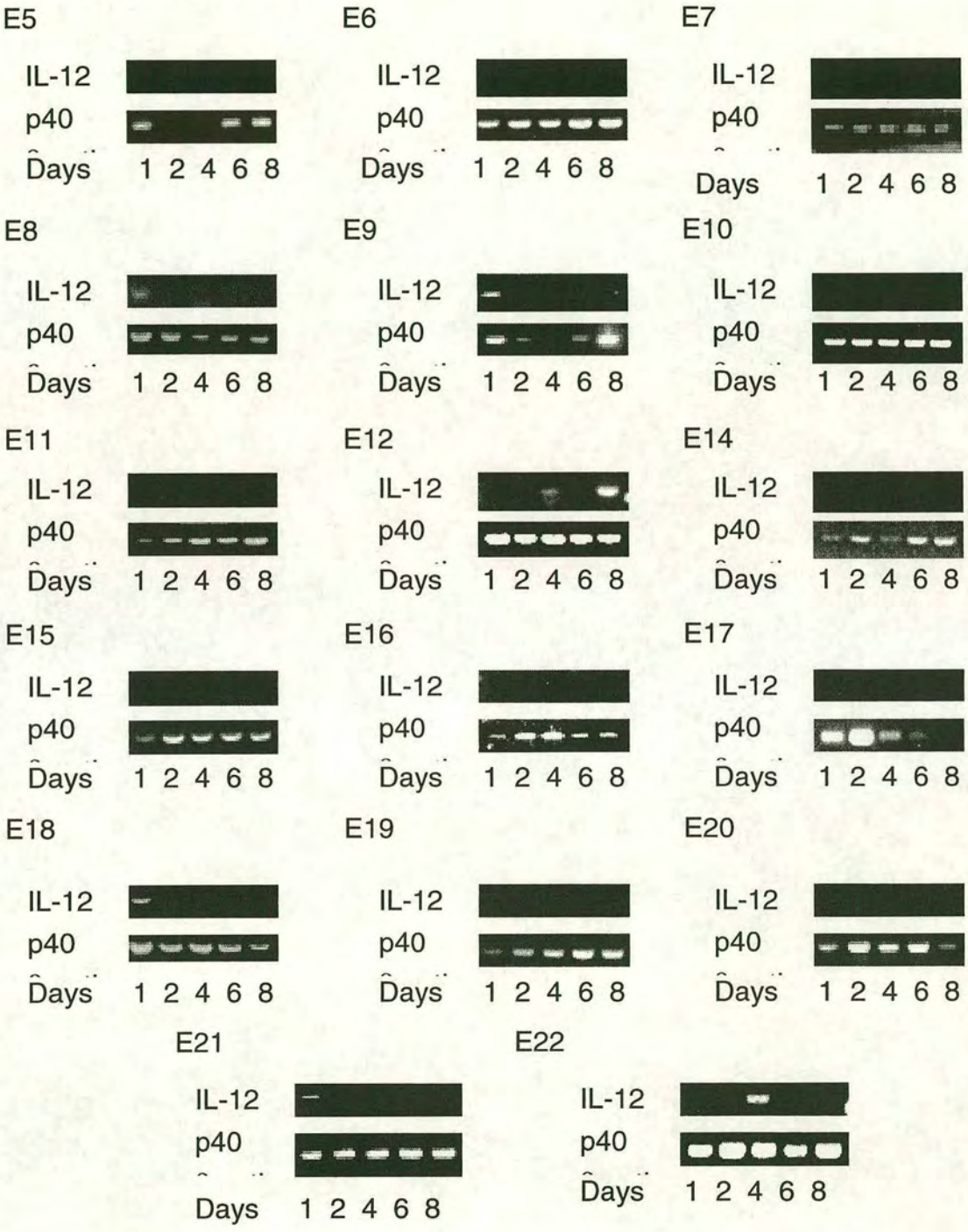


Table 4.2: Summary of the number of individuals who had detectable IL-12 p40 transcripts in response to uRBC, PfSE and LPS (or PHA for naives) in a) naïve (n=13, n=11 for LPS/PHA) and b) exposed (n=20) study populations.

a) Naives

	Stimulus		
Day	**RBC**	**PfSE**	**LPS/PHA**
1	4 (31%)	5 (38%)	6 (55%)
2	5 (38%)	6 (46%)	8 (73%)
4	6 (46%)	10 (77%)	8 (73%)
6	6 (46%)	10 (77%)	10 (91%)

b) Exposed

	Stimulus		
Day	**RBC**	**PfSE**	**LPS**
1	4 (20%)	4 (20%)	7 (35%)
2	4 (20%)	4 (20%)	7 (35%)
4	4 (20%)	6 (30%)	7 (35%)
6	4 (20%)	6 (30%)	7 (35%)
8	4 (20%)	6 (30%)	7 (35%)

Table 4.3: Comparison between RT-PCR and ELISA in measuring IL-12 p40 for each naïve individual. IL-12 p40 protein was measured on day 2 and compared with cumulative transcript responses to a) uRBC and b) PfSE (IL-12 p40 protein levels were considered positive if they were above the LLD =171pg/ml).

a) uRBC

Donor	**RT-PCR**	**ELISA**
N2	-	-
N3	-	+
N4	+	+
N5	-	+
N12	+	+
N13	+	+
N14	-	+
N15	-	-
N16	+	-
N17	+	+
N18	-	+
N19	-	+
Sum	**5**	**9**
%	**42%**	**75%**
Ratio	**5/12**	**9/12**

b) PfSE

Donor	**RT-PCR**	**ELISA**
N2	+	-
N3	-	+
N4	+	+
N5	-	+
N12	+	+
N13	+	+
N14	+	+
N15	+	+
N16	-	+
N17	+	+
N18	+	+
N19	+	+
Sum	**9**	**11**
%	**75%**	**92%**
Ratio	**9/12**	**11/12**

Table 4.4: Comparison between RT-PCR and ELISA in measuring IL-12 p40 for each exposed individual. IL-12 p40 protein was measured on day 2 and compared with cumulative transcript responses to a) uRBC and b) PfSE (IL-12 p40 protein levels were considered positive if they were above the LLD =171pg/ml).

a) uRBC

Donor	RT-PCR	ELISA
E1	-	+
E2	-	+
E3	-	+
E5	-	+
E6	-	+
E7	-	+
E8	-	+
E9	+	+
E10	-	+
E11	+	+
E12	+	+
E14	-	+
E15	-	+
E16	-	+
E17	-	+
E18	+	+
E19	-	+
E20	-	-
E21	-	+
E22	-	+
Sum	**4**	**19**
%	**20%**	**95%**
Ratio	**4/20**	**19/20**

b) PfSE

Donor	RT-PCR	ELISA
E1	-	+
E2	-	+
E3	-	+
E5	-	+
E6	-	+
E7	-	-
E8	+	+
E9	+	+
E10	-	+
E11	-	+
E12	+	+
E14	-	+
E15	-	+
E16	-	+
E17	-	-
E18	+	+
E19	-	+
E20	-	-
E21	+	+
E22	+	+
Sum	**6**	**17**
%	**30%**	**85%**
Ratio	**6/20**	**17/20**

Table 4.5: Comparison of RT-PCR and ELISA methods in detecting IL-12 p40 responses to uRBC and PfSE in a) naïve (n=12) and b) exposed (n=20) study populations. Number (%) of responders detected by each method.

a) Naives

	Ratios	
	RT-PCR	**ELISA**
RBC	5 (42%)	9 (75%)
PfSE	9 (75%)	11 (92%)

b) Exposed

	Ratios	
	RT-PCR	**ELISA**
RBC	4 (20%)	19 (95%)
PfSE	6 (30%)	17 (85%)

Chapter 5: Naïve, exposed and immune individuals have lymphoproliferative responses to *P. falciparum* antigen *in vitro*

5.1 Introduction

Both T and B cell responses play important roles in protection to malaria. The primary focus used to be on humoral responses, because it was found that malaria-specific Ab could be induced during an immune response. It was originally thought that these responses were protective and thus they pointed the way towards vaccine therapy. It was then realised that although these Ab-mediated responses were sometimes protective, they did not give complete sterilising immunity. For example, Hoffman *et al.* demonstrated that individuals who had high levels of Ab to sporozoites could still become infected with malaria (139).

Because T cells regulate both cell-mediated and Ab-dependent immune responses, there has been a major focus on elucidating T cell mechanisms in immunity to malaria. Understanding more about the role of T cell responses is fundamental to an understanding of protective immunity. A number of studies have focused on the need for vaccines to stimulate specific T cells which will clonally expand and either directly or indirectly destroy the invading organism (77, 324).

5.1.1 T cell responses in malaria-exposed individuals

Individuals who live in endemic areas are continuously exposed to malaria. When cultured *in vitro*, their cells respond vigorously to malaria Ag (114, 284, 336, 366). Some of these responses are still found long after initial exposure, indicating that there is long-lasting memory to malaria (73)

However, there have also been studies which show that malaria-exposed individuals have low or absent proliferative responses *in vitro* to malaria Ag, particularly during acute infection (284). Luty *et al.* found that individuals living in Gabon who had higher levels of parasitemia had lower proliferative responses (211). Ho *et al.* were also unable to demonstrate proliferative responses to malaria Ag *in vitro* from cells of splenectomised patients with acute malaria (136). It was demonstrated that there is an absence of circulating malaria-specific T cells in the periphery during acute infection, because activated cells upregulate LFA-1 and most likely sequester in the spleen or

liver during infection (146). It was also suggested that malaria Ag themselves could be immunosuppressive. Riley *et al.* demonstrated that *P. falciparum* extracts suppressed proliferative responses to Ag and mitogen *in vitro*, suggesting that not just malaria Ag-specific responses are suppressed (285).

5.1.2 T cell responses in unsensitised individuals

Studies done in the late 1970s-early 1980s demonstrated that PBMCs, primarily T cells, from individuals who have never been exposed to malaria as well as cells from exposed individuals proliferate in response to *P. falciparum* extracts (14, 20, 62, 63, 97, 105, 114, 115, 119, 290, 366, 370). Because cells from naive individuals were also able to respond, it was thought at the time that *P. falciparum* extracts were mitogenic (119). However, more recent studies showed that T cell responses to malaria Ag were MHC-dependent, meaning that malaria parasites could be activating via a superantigen- or Ag-mediated mechanism (see section 1.3)(20, 62, 63, 114, 115, 290, 370).

The general consensus is that *P. falciparum* extracts are acting as conventional Ag. A number of studies demonstrated that T cell responses to *P. falciparum* are not only MHC-dependent but are MHC-restricted (97, 114, 290). It is generally agreed that PBMCs from naive individuals respond because their cells have been primed previously by other environmental Ag which share similar antigenic epitopes. Some studies have demonstrated that there is a rapid *in vitro* expansion of *P. falciparum*-specific CD4+ $\alpha\beta$+ CD45RO+ memory T cells in individuals who have had no prior exposure to malaria (62, 63, 75). Clones generated from these memory T were able to proliferate in response to a number of common environmental Ag such as *Streptococcus pyogenes, Alternaria, Aspergillus* and *Epidermophyton* (62, 63).

5.1.3 Is lymphoproliferation an indicator of immunity?

Since both sensitised and unsensitised individuals both respond to malaria *in vitro*, proliferative responses may not be indicative of whether immune responses lead to pathogenesis or protection. However, the results of T cell activation may be different between naïve and exposed populations. T cell responses in individuals who are immune are Ag-specific, activating only a specific subset of cells. In contrast, cells from

naïve individuals may be responding to a number of antigenic epitopes, primed by cross-environmental Ag, resulting in a large proliferation of T cells.

Lymphoproliferative responses to malaria Ag have mainly been studied in naïve and acutely infected individuals. Less is known about the differences in cell mediated immunity between clinically immune and naïve individuals (284). Here I present a study in which the proliferative responses to crude malaria Ag of cells from naïve, exposed and malaria-immune individuals were analysed and compared.

5.2 Materials and methods

5.2.1 Study population and cell culture

Ten to 30 ml of venous blood was taken from 19 naïve individuals, 20 exposed individuals and 20 immune individuals, and PBMCs were isolated *for in vitro* cell cultures (see section 2.1.1 for description of study populations and section 2.4 for method of cell separation). Samples from 4 additional naïve individuals were used as controls when setting up cultures in Ghana. Cultures were set up in triplicate in round-bottomed 96-well microtitre plates. PBMCs were distributed at 10^5 cells/well in a total culture volume of 200 µl and were cultured for 2, 3, 5, 7 and 9 days with uRBC or PfSE. PHA (2 µg/ml) was used as a positive control for proliferation (concentrations of stimuli are give in sections 2.2.3 and 2.3).

Prior exposure and use of anti-malarial drugs was determined by a questionnaire completed by each donor. An aliquot of plasma was removed for anti-malarial Ab measurements, and parasite detection for all African donors was determined by Giemsa-stained blood films and PCR. Details of the serology and PCR methods are given in sections 2.1.3 and 2.1.2 respectively.

5.2.2 Lymphocyte proliferation assay

Eighteen to 20 hours before cells were harvested, 100 µl of supernatant were removed from each well and reserved for cytokine analysis. Cultures were then pulsed with 1 µCi radioactive [methyl-^{3}H] thymidine in 100 µl of fresh culture medium per well. Cells were harvested onto filter mats and dried overnight. Filter mats were either read directly on a dry beta counter, or scintillation fluid was added and read with a

scintillation counter. [methyl-^{3}H] thymidine incorporation was assayed for 1 minute in counts per minute (see section 2.5 for a complete description of materials used for proliferation assays).

5.2.3 Data analysis

The geometric mean cpm for triplicate wells was calculated and expressed as mean cpm. The SI for each sample was calculated as the mean cpm for PfSE or PHA cultures divided by the mean cpm for uRBC cultures. SI values > 2.5 were considered positive (27).

Comparisons within each group for uRBC versus PfSE were assessed by paired t test on log transformed mean cpm. Because the beta counters used in Ghana and in the UK measured emissions in linear and logarithmic fashion respectively, it was not possible to directly compare cpm values counted in different places. Therefore, differences between groups were assessed by student's t test on log transformed SI. Chi-squared analysis was performed to compare responders versus non-responders (i.e. SI $\geq$ 2.5). Correlations between proliferation and Ab responses were assessed with Pearson correlation test.

5.3 Results

5.3.1 Study populations

The three groups of donors were clearly distinguishable on the basis of their clinical history, malaria infection rates and serological responses to soluble malaria antigens (see tables 2.1a-c and 2.2, Chapter 2). The naive donors had no previous history of malaria infection and antibody levels were within the normal range defined by non-immune sera. The majority of the exposed, but non-immune donors reported a clinical malaria infection (confirmed by microscopy and treated with anti-malarial drugs) within the last five years. Only one of the donors had a subclinical malaria infection at the time of blood sampling. Several donors had anti-malarial antibody levels below the cut-off level defined by non-immune sera and the mean antibody titre was only 41% of the hyperimmune control serum. In contrast, in the clinically immune group, only one donor reported a confirmed clinical malaria attack in the past five years, 8 donors were

subclinically infected at the time of sampling (two detected by blood film, and 6 by PCR), all donors were seropositive and mean antibody titers were 87% of the hyperimmune control.

5.3.2 Study populations' responses to PfSE

Proliferative responses were measured after 3, 5, 7, and 9 days for naïve individuals, after 2, 3, 5, 7, and 9 days for exposed individuals and after 3, 5 and 7 days for immune individuals (a limited amount of blood was allowed to be taken for immune donors). Figure 5.1 shows the proliferative responses to PfSE for each individual over the timecourse of the assay, and table 5.1 shows the geometric mean cpm and 95% confidence intervals of proliferative responses to uRBC and PfSE (complete details for each individual are given in Appendix 2, tables A2.1-4). Cells from all donors proliferated strongly to PHA, showing that cells were viable (data not shown, see Appendix 2, tables A2.1-4). In all 3 groups proliferative responses to PfSE increased over time although responses started to decrease slightly by day 9. There were significant differences between proliferation to uRBC and to PfSE on all days for each of the groups (paired $t \geq 3.99$, $p < 0.001$, $df = 18$ for naives, paired $t \geq 7.48$, $p < 0.001$, $df \geq 18$ for exposed, paired $t \geq 6.36$, $p < 0.001$, $df \geq 11$ for immunes).

Because 8/20 immune donors were subclinically infected at the time of blood sampling, maximum proliferative responses between non-infected and infected individuals were compared. Although proliferation to PfSE was higher in non-infected individuals (geometric mean = 2053 and geometric mean = 892 for infected individuals), the difference was not significant (student $t = 1.22$, $p>0.05$, $df=26$).

5.3.3 Differences in proliferative responses between groups

Proliferative responses were compared on days 3, 5 and 7. In all 3 groups, the geometric mean SI for PfSE/uRBC increased steadily with time (figure 5.2), but there was no significant difference between the groups in either the mean SI ($SI \geq 2.5$, paired $t \leq 1.62$, $p > 0.05$, $df \geq 29$) or in the proportion of responders. For example, at day 7, cells from 19/19 naive, 19/19 exposed and 18/20 immune donors gave positive responses to PfSE. The only noticeable difference between the groups was that

responses appeared slightly earlier in the exposed group than in the other two groups (table 5.2). Thus, cells from 13/19 donors gave an SI ≥ 2.5 at day 3 compared to 6/19 naives and 8/20 immunes, but the difference was not significant ($\chi^2 = 4.46$, $p > 0.05$, $df = 2$).

5.3.4 Correlation between proliferative and antibody responses

Anti-malarial Ab responses were significantly correlated with maximum proliferative responses in exposed individuals (figure 5.3, $r^2 = 0.43$, $p = 0.002$). However, there was no correlation found between proliferation and Ab responses for naïve or immune individuals ($r^2 = 0.12$, $p > 0.05$ for naïves and $r^2 = 0.17$, $p > 0.05$ for immunes).

Discussion

5.4.1 Study populations

Subjects were allocated to naive, exposed or immune groups according to their place of residence and before any laboratory analyses (parasite detection, serology or cellular assays) were performed. Parasite detection and serology confirmed our allocation of individuals to appropriate groups, with the possible exception of one person in the exposed group (who had a low density, asymptomatic infection at the time of sampling and might therefore be regarded as clinically immune) and one person in the immune group (who reported a clinical attack of malaria 9 months previously and might therefore be considered exposed but non-immune). However, to avoid selection bias, neither of these subjects was reallocated to alternative groups.

5.4.2 Lymphoproliferation and immunity to *P. falciparum.*

PBMCs from naïve, exposed and immune individuals responded vigorously to PfSE *in vitro.* However, there was no significant difference in proliferative responses between any of the groups. Thus, lymphoproliferative responses are not associated with clinical immunity in this study. These results have been confirmed by other studies (283).

It might be expected that immune and exposed individuals would have higher proliferative responses, because their immune systems have already been primed

specifically to *P. falciparum*, and malaria specific clones will have been expanded. Thus there should be a higher precursor frequency of malaria reactive cells. Some studies have demonstrated that sensitised individuals do show higher responses to *P. falciparum* Ag (14, 366). However, proliferative responses depend on a number of factors, such as the type of Ag. A crude schizont extract was used as an Ag in this study, but other studies have used different preparations of malaria Ag which result in differences of cellular subset expansion (354) and in differences in proliferative responses between individuals of varying degrees of immunity (284). Riley *et al.* 1988 showed that PBMCs from naïve individuals did not respond *in vitro* to soluble malaria antigens whereas those from immune individuals did (284). Further, Ballet *et al.* 1981 demonstrated that proliferative responses depended upon the concentration of Ag put into culture. A higher concentration of *P. falciparum* added to lymphocyte cultures resulted in similar proliferative responses for naïve and exposed individuals, whereas a lower concentration resulted in lower proliferation for naïve, but not exposed, individuals (14). Cross-reactive Ag may only be present in relatively low concentrations. Thus, a lot of Ag may be needed to stimulate cells from naïve donors.

However, naive individuals also have strong proliferative responses, most likely resulting from being previously sensitised by cross-environmental antigens. Goodier *et al.* found much higher proliferative responses to malaria Ag in non-exposed versus exposed individuals (115). In previous studies on a group of immune individuals from the Gambia (Dick *et al.*, unpublished data), no significant difference was found in the group mean SI when comparing these immune individuals to naives. Although there were more low responders in the immune group, their stimulation indices were in a similar range as naïve individuals.

5.4.3 What do lymphoproliferative responses indicate?

The selection of peripheral blood as the source of leucocytes is dictated by practical constraints but has been validated in many previous studies: although malaria-reactive T cells tend to disappear from the peripheral circulation during an acute infection (probably migrating to the spleen and liver) they are released back into the periphery upon resolution of the infection (146). Although some of the immune individuals had subclinical malaria infection at the time of blood sampling, their PBMCs

still responded vigorously to PfSE, indicating that these individuals still have malaria reactive lymphocytes in their peripheral circulation. It is thus realistic to assume that, in healthy individuals, the peripheral T cell population is representative of the total malaria-reactive T cell pool.

However, lymphocyte proliferation assays do not indicate cell function or cell phenotype. It could be hypothesised that although there is no difference in whole PBMC proliferative responses, there may be a difference in which subsets of cells are being activated (see Chapter 7) and what cytokines are being produced (see Chapter 6). There was a significant correlation between anti-malarial Ab and maximum lymphoproliferative responses in exposed individuals. Although there was no significant correlation found between Ab responses and lymphoproliferation in immune individuals, both responses were high, indicating that cells proliferating in these individuals may be Th2 cells providing help to generate an Ab-mediated response.

In conclusion, lymphoproliferation is not an indicator of clinical immunity. However, the strong lymphoproliferative responses in all 3 study populations demonstrate that all groups have populations of primed T cells able to recognise malaria Ag. However, the clear differences in clinical status between the groups indicate that there may be differences in the cellular subsets of responding cells and their cytokine production profile, which explain differences in susceptibility to clinical malaria.

Figure 5.1: Geometric mean cpm values to PfSE of PBMCs for each individual over a 9 day timecourse for a) naïve b) exposes and c) immune study populations (7 day timecourse for immune individuals). Ranges of cpm for uRBC-stimulated cultures for all days are 22-5567 for naïves, 15-1660 for exposed and 16-1151 for immunes.

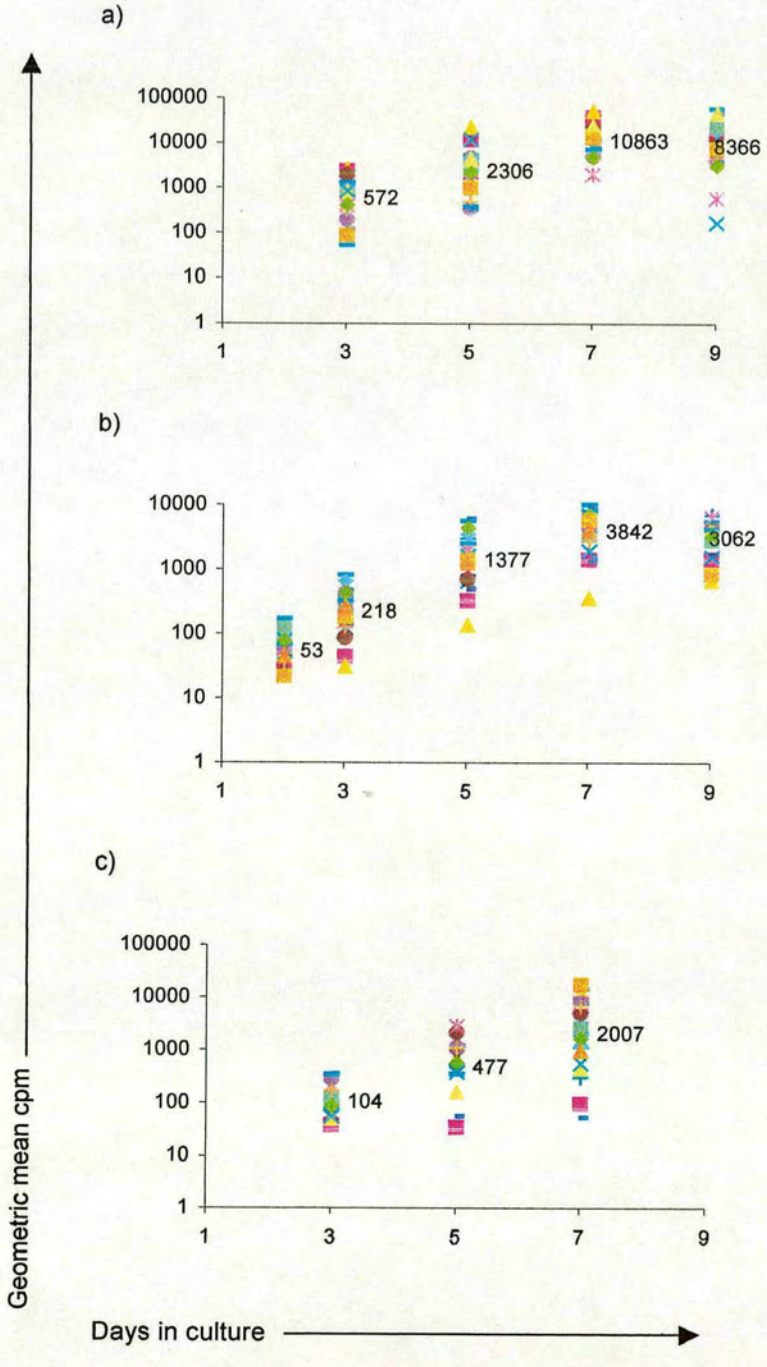


Table 5.1: Geometric mean cpm and 95% CI to PfSE in PBMCs from a) naïve b) exposed and c) immune individuals for each day.

a)

	uRBC-stimulated		PfSE-stimulated				
Day	**Mean cpm**	**CI**	**Mean cpm**	**CI**	**t**	**p**	**n**
3	238	136, 416	572	332, 984	3.99	< 0.001	19
5	308	163, 581	2306	1280, 4155	8.86	< 0.001	19
7	663	408, 1077	10863	7486, 15763	16.03	< 0.001	19
9	909	485, 1704	8366	4102, 17059	7.15	< 0.001	19

b)

	uRBC-stimulated		PfSE-stimulated				
Day	**Mean cpm**	**CI**	**Mean cpm**	**CI**	**t**	**p**	**n***
2	30	24, 39	53	41, 68	7.48	< 0.001	20
3	61	46, 79	218	146, 325	10.62	< 0.001	20
5	153	111, 211	1377	880. 2153	18.56	< 0.001	19
7	275	191, 397	3842	2635, 5602	15.94	< 0.001	19
9	457	288, 727	3062	1534, 4596	11.39	< 0.001	16

c)

	uRBC-stimulated		PfSE-stimulated				
Day	**Mean cpm**	**CI**	**Mean cpm**	**CI**	**t**	**p**	**n***
3	36	25, 51	104	76, 147	6.36	< 0.001	18
5	60	32, 111	477	215, 1060	6.50	< 0.001	12
7	107	67, 171	2007	924, 4363	8.30	< 0.001	20

* there were insufficient cells from some individuals to set a full set of cultures.
cpm- counts per minute.
CI- confidence interval.
t- t value for the significance level.
p- p value.
n- number of samples/individuals on given day.

Figure 5.2: Lymphoproliferative responses to PfSE of PBMCs from naïve (■), exposed (▲) and immune (●) donors expressed as geometric mean SI (PfSE/uRBC) with 95% CI.

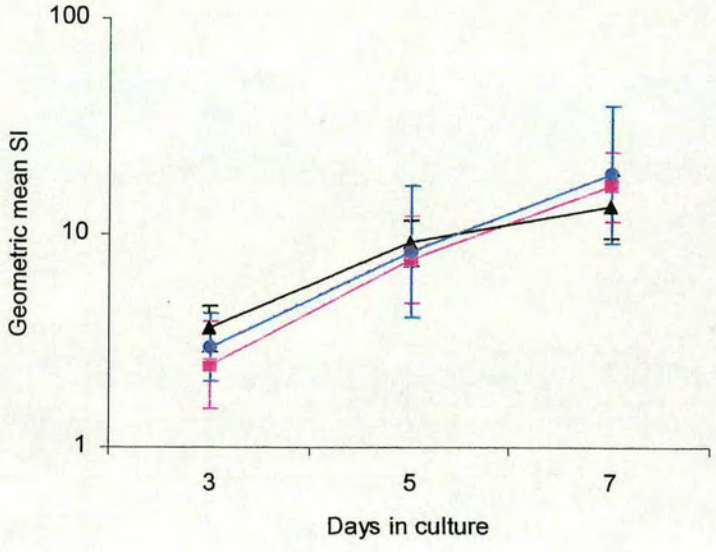
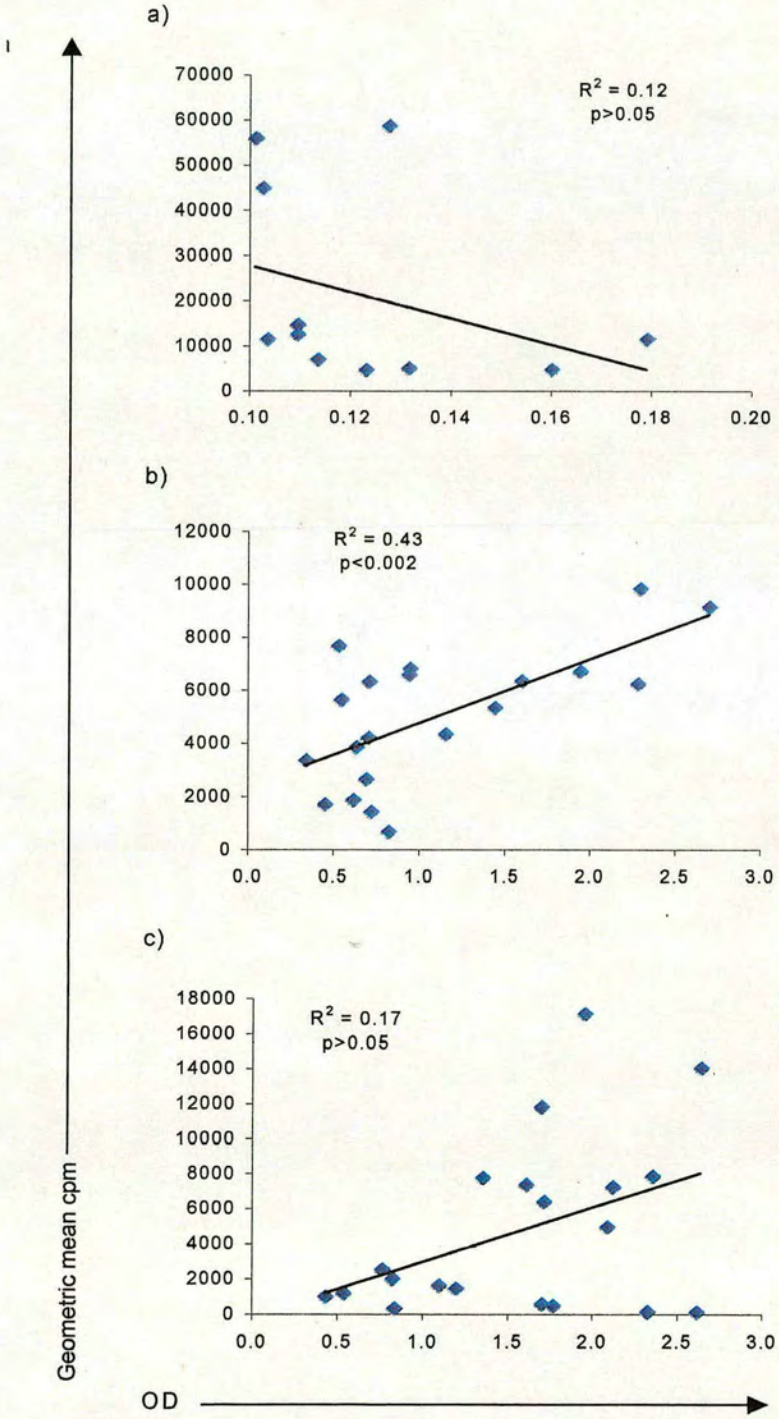


Table 5.2: Number of positive responders to PfSE in naïve, exposed and immune donors (positive responder determined as SI (PfSE/uRBC) ≥ 2.5).

Day	Naives	Exposed	Immunes
3	6/19 (32%)	13/19 (65%)	8/18 (44%)
5	18/19 (96%)	19/19 (100%)	11/12 (92%)
7	19/19 (100%)	19/19 (100%)	18/20 (90%)

Figure 5.3: Correlation of anti-malarial Ab responses with maximum geometric mean cpm for a) naïve (n=11) b) exposed (n = 20) and c) immune individuals (n = 20).



Chapter 6: Downregulation of IL-12-dependent and IL-12-independent IFN-γ production is associated with acquisition of clinical immunity to *P. falciparum* malaria[1]

6.1 Introduction

Individuals living in malaria endemic areas eventually develop clinical immunity to *Plasmodium falciparum*, meaning, that there is an absence of clinical symptoms (i.e. fever, malaise) even in the presence of parasites in the bloodstream. Pro-inflammatory cytokines aid in parasite killing (98, 216, 310), but clinical symptoms of malaria infection are also a result of an excessive inflammatory response (67, 181, 293). However, it is not clear whether clinical immunity develops as a result of anti-parasitic mechanisms or whether anti-toxic immunity (downregulating an excessive inflammatory response) is also involved.

6.1.1 The role of inflammatory cytokines in malaria infection

Studies in mice demonstrate that IFN-γ is essential in clearing primary malaria infections (70, 93). Early IFN-γ production from NK cells and T cells has been shown to mediate resistance to *P. c. chabaudi* (236) and *P. yoelii* infections (9) respectively. IL-12 induced IFN-γ production mediates resistance via NO (151, 309, 320) and oxygen intermediates (i.e. H_2O_2) (310) which may be involved in parasite killing (286, 310). However, IFN-γ also plays a role in the acute symptoms of infection, such as fever, anorexia and weight loss, through induction of TNF-α and other endogenous pyrogens. Over-production of IL-12, IFN-γ or TNF-α predisposes to severe pathology (152, 181, 350, 368) and is associated with cerebral malaria (67, 293, 367).

In humans, elevated serum IFN-γ levels were found in convalescent individuals during parasite clearance (363) and in individuals who did not present with malaria (versus those who did)(74), suggesting a beneficial role in ihibiting parasite replication. Similarly, plasma TNF-α and nitrogen oxide levels are associated with rapid resolution of fever and parasite clearance (182, 183). However, elevated serum levels of inflammatory cytokines are also associated with severe *P. falciparum* malaria (15, 118, 193, 238). Additionally, IFN-γ responses to certain malaria antigens correlate with

[1] See Appendix 5, manuscript submitted for publication.

predisposition to clinical symptoms (283). Taken together, these observations suggest that inflammatory cytokines are involved both in the pathogenesis and in the immunity to malaria infection.

Previous studies show that PBMCs from malaria immune individuals produce very little IFN-γ in response to crude malaria Ag (53, 284). In contrast, cells from individuals who have never been exposed to malaria, produce significant amounts of IFN-γ to schizont extracts or live malaria parasites (75, 354), suggesting that cross-reactive memory T cells, primed by other common environmental organisms, are responding (62).

6.1.2 The role of anti-inflammatory cytokines in malaria infection

Work in mouse malaria models suggests that an early inflammatory (Th1) response reduces parasitemia (96, 330), but a switch to a Th2 response, with development of malaria-specific antibodies and production of IL-4, is required for clearance of infection (196, 197). Adding exogenous anti-inflammatory cytokines, such as IL-10 and TGF-β, reduced mortality in mice against *P. berghei* infection (180, 252). Non-lethal infections of *P. yoelii* and *P. berghei* in resistant strains of mice are correlated with higher circulating levels of TGF-β in serum and increased IL-10 mRNA transcripts in the spleen (180, 252). It is thought that these cytokines decrease Mø and NK cell production of inflammatory cytokines, IL-12, IFN-γ and TNF-α (206, 346).

In humans, decreased circulating levels of TGF-β were found in individuals with acute *P. falciparum* infection and TGF-β levels were inversely correlated with serum levels of TNF-α (355). Malaria-immune individuals had higher ratios of IL-4/IFN-γ-producing cells which were correlated with elevated serum levels of malaria-specific IgE (82). Ho *et al.* demonstrated that IL-10 decreased malaria-induced pro-inflammatory cytokine production (134). However, higher levels of serum IL-10 were found in individuals who had severe *P. falciparum* infection versus individuals with uncomplicated infection (15, 135, 154, 263, 356), suggesting that there is a critical balance in timing and concentration of cytokine production that determines clinical immunity to malaria.

6.1.3 Hypothesis

I hypothesise that there is an association between *in vitro* levels of PfSE-stimulated IFN-γ production and the risk of clinical malaria. In order to test this hypothesis, I have compared IFN-γ levels of PfSE-stimulated PBMCs from naive, exposed (but not clinically immune) and immune donors. I have also measured PfSE-stimulated IL-12 and IL-10 levels and examined the effect of neutralising αIL-12 on IFN-γ production, in order to determine how IFN-γ production is regulated.

6.2 Materials and methods

6.2.1 Study population and cell culture

Ten to 30 ml of venous blood were taken from 19 naïve individuals, 20 exposed individuals and 20 immune individuals and processed for *in vitro* PBMC cultures (see section 2.1.1 for description of study populations and section 2.4 for method of cell separation). Blood from 4 additional naïve individuals was used as controls when setting up cultures in Ghana. PBMCs were distributed at 10^6/well in flat-bottomed 24-well plates or in triplicate at 10^5/well in round-bottomed 96-well microtitre plates in a total culture volume of 1 ml and 200 µl respectively.

Cells were cultured for 1, 2, 4, 6 and 8 days with uRBC or PfSE. PPD (only for immunes) and PHA were used as positive controls for IFN-γ production. PHA and LPS plus recombinant IFN-γ were used as positive controls for IL-12 production. In order to determine the relationship between IL-12 and IFN-γ, neutralising αIL-12 or a control isotype-matched goat IgG were also added to cultures (concentrations of reagents are given in sections 2.2.3 and 2.3). Three hundred to 900 µl of culture supernatant were harvested at various timepoints and stored at -70°C until tested for cytokine levels.

Lymphocyte proliferation assays were also performed for the above cultures (see sections 2.5 and 5.2.2). IL-12 p40 production by PBMCs stimulated with uRBC, PfSE, PHA or LPS was also assessed by RT-PCR (see Chapter 4).

6.2.2 Cytokine assays

IFN-γ, IL-12 p70, IL-12 p40, and IL-10 were measured in cell culture supernatants by 2-site capture ELISA. Immulon 4, 96-well, flat-bottom microtitre plates were coated with an optimal concentration of coating Ab (see Chapter 3, table 3.2 for

reagent concentrations and buffer/wash solutions) and incubated at RT overnight. Plates were washed, blocked with blocking buffer for 1 hour at 37°C, and then washed again (see Chapter 3, table 3.1 for basic ELISA protocol). Standards or culture supernatant were added to duplicate wells and incubated at RT for 2-3 hours. After plates were washed, an optimal concentration of biotinylated capture Ab was added and incubated for 1 hour at RT. Plates were washed, avidin-labelled horseradish peroxidase was added for thirty minutes at RT, plates were washed a final time, and were developed using OPD and H_2O_2 as chromogen/substrate. The reaction was stopped with 2 M H_2SO_4 after 15 minutes, and absorbance was read at 492 ηm.

6.2.3 Data analysis

Cytokine concentrations were calculated from best fit curve formula based on linear regression analysis. The LLD for each assay was as follows: IFN-γ 44-96 pg/ml, IL-12 p70 107 pg/ml, IL-12 p40 171 pg/ml, and IL-10 47 pg/ml.

Comparisons within each group for uRBC versus PfSE were assessed by paired t test on log transformed cytokine concentrations. Differences between groups for IFN-γ and IL-12 p40 (PfSE-uRBC) concentrations were assessed by non-parametric Mann Whitney U test. Student's t test on log transformed data was used to compare IL-10 values. Chi-squared analysis was performed to compare the proportion of responders versus non-responders. Responders were defined as the following: for IFN-γ, twice as much IFN-γ from PfSE-stimulated cultures as from uRBC-stimulated cultures; for IL-12 p40, IL-12 p40 from PfSE-stimulated cultures minus IL-12 p40 from uRBC-stimulated cultures ≥ 20 pg/ml; for IL-10, IL-10 from PfSE-stimulated cultures > IL-10 from uRBC-stimulated cultures and > LLD. Correlations between lymphoproliferative responses and each cytokine response were assessed by the Pearson correlation test.

6.3 Results

6.3.1 IFN-γ production

6.3.1.1 PBMCs from all groups of donors produce significant levels of IFN-γ

IFN-γ in cell supernatants was measured after 1, 2, 4, 6 and 8 days for naïve and exposed individuals and after 2, 4, and 6 days for immune individuals (only a limited amount of blood was available for immune individuals). Table 6.1 shows the geometric

mean and CI for IFN-γ responses to uRBC and PfSE (complete details for each donor are given in Appendix 3, tables A3.1-A3.4). Cells from all donors produced IFN-γ in response to PHA (maximum values were 12493 pg/ml for naives, 10701pg/ml for exposed individuals and 10952 pg/ml for immune individuals). PBMCs from naïve individuals produced moderate amounts of IFN-γ, which steadily increased over time and peaked by day 6 (224 pg/ml). IFN-γ levels in PfSE cultures were significantly higher than in uRBC cultures on days 4, 6 and 8 (paired $t \geq 3.6$, $p \leq 0.002$, $df \geq 12$ on all days). PfSE-stimulated PBMCs from exposed individuals produced significantly higher levels of IFN-γ than uRBC controls on all days and peaked at 1069 pg/ml on day 8 (paired $t \geq 2.43$, $p \leq 0.03$, $df \geq 17$ on all days). In contrast, PBMCs from immune donors produced much lower levels of IFN-γ in response to PfSE (maximum IFN-γ level on day 6, 91pg/ml), although mean IFN-γ levels were significantly higher than in uRBC-stimulated cultures on days 4 and 6 (paired $t \geq 2.75$, $p \leq 0.02$, $df \geq 12$).

6.3.1.2 Immune donors produce minimal amounts of IFN-γ

Median IFN-γ levels (PfSE-uRBC) were compared between groups on days 2, 4 and 6 (figure 6.1). PFSE-stimulated PBMCs from exposed individuals produced significantly higher amounts of IFN-γ than naïve donors on days 4 and 6 (Mann-Whitney U, $W = 253$, $p \leq 0.001$, $df \geq 35$). Immune donors had significantly lower median IFN-γ levels than in the naive group on days 4 and 6 (Mann-Whitney U, $W \geq 384$, $p \leq 0.008$, $df \geq 30$) and in the exposed group on days 2, 4 and day 6 (Mann-Whitney U, $W \geq 230$, $p \leq 0.003$, $df \geq 30$).

However, not all PBMCs from naïve donors produced IFN-γ. Additionally, PBMCs from a number of immune donors did produce IFN-γ, although IFN-γ levels were not as high as for exposed individuals. The number of IFN-γ responders (PfSE ≥ 2XuRBC IFN-γ on any day) was highest for exposed donors (19/20, 95%), intermediate for naïve donors (14/19, 74%) and lowest for immune donors (5/20, 25 %)(table 6.2).

6.3.1.3 Correlation between proliferative and IFN-γ responses

In vitro IFN-γ production for each group was compared with the kinetic timecourse of proliferative responses on the same day. The kinetics of median IFN-γ responses are similar to changes in mean SI up to day 7, in all 3 groups (figure 6.2,

values are given in Appendix 2, table A3.5). By day 9, proliferative responses were decreasing while IFN-γ responses were increasing for naïve individuals (figure 5.4a). There were also significant positive correlations between individual proliferative and IFN-γ responses for donors in all 3 groups (figure 6.3, $r^2 = 0.13$, $p = <0.001$ for naives, $r^2 = 0.33$ $p < 0.001$ for exposed and $r^2 = 0.57$, $p < 0.001$ for immunes). However, when responses from all 3 groups were combined, there was no significant correlation between proliferative and IFN-γ responses ($r^2 = 0.01$, $p > 0.05$), because the IFN-γ responses were low in immune individuals and high in exposed individuals. Naïve and exposed individuals were more likely to have positive proliferative and IFN-γ responses to PfSE (14/19 and 19/20 respectively), but most immune individuals had negative IFN-γ responses although they had strong proliferative responses (13/20).

6.3.2 IL-12 production

6.3.2.1 IL-12 levels in culture supernatants

IL-12 was initially measured by IL-12 p70 ELISA. PBMCs from 3 naïve donors were stimulated with varying concentrations of PfSE ($3.1X10^5$ parasites/ml to $3.9X10^4$ parasites/ml), and cell culture supernatants were collected at various timepoints (4 to 115 hours). IL-12 p70 levels were below LLD for almost all of the timepoints collected (see Appendix 3, table A3.6).

Because it was thought that the IL-12 p70 ELISA was not sensitive enough, IL-12 p40 transcripts were measured by RT-PCR and free IL-12 p40 was measured by IL-12 p40 ELISA. IL-12 p40 was measured on days 1, 2, 4, 6 and 8 of PfSE-stimulated cultures for naïve and exposed individuals and on days 2, 4 and 6 for immune individuals (only ELISA detection for immunes). Detection of IL-12 p40 transcripts was not consistent (see Chapter 4). For the IL-12 p40 ELISA, IL-12 levels were highest on day 2, therefore only day 2 data is shown (table 6.3, see Appendix 3, table A3.7 for complete data on each individual).

Overall, levels of PfSE-specific IL-12 in supernatants were very low. There was no significant difference between uRBC and PfSE stimulated cultures in all 3 groups (paired $t \leq 1.93$, $p > 0.05$, $df \geq 17$). PBMCs from exposed donors produced up to 530 pg/ml IL-12 (PfSE-uRBC), but levels varied widely between individuals. Median IL-12 levels were not significantly different between the groups (figure 6.4, Mann Whitney U

$W \geq 289$, $p > 0.05$, $df \geq 36$). When individuals are categorised as responders or non-responders (PfSE-uRBC IL-12 ≥ 20 pg/ml and > LLD), 11% (2/18) of naïve donors 45% (9/20) of exposed donors and 35% (7/20) of immune donors were responders responded, but the difference was not significant ($\chi^2 = 5.31$, $p > 0.05$, $df = 2$).

6.3.2.2 Relationship between IL-12 and IFN-γ production.

Although IL-12 p40 levels in supernatants were low, significant amounts of IL-12 p40 were detected in culture supernatants from some donors. Thus, the kinetic relationship between IL-12 and IFN-γ production was compared in individual donors. IL-12 production normally peaked at day 1 or 2 before there was an increase of IFN-γ production (figure 6.5). However, there was no overall correlation between IL-12 levels and IFN-γ production for any of the groups (data not shown). Thus in order to determine whether there was a causal relationship between IL-12 production and IFN-γ production, neutralising αIL-12 or control IgG was added to PfSE-stimulated or uRBC-stimulated cell cultures and IFN-γ levels were measured.

Although αIL-12 or control IgG had some effect on lymphoproliferative responses (mean SI), most differences were not significant, and αIL-12 did not consistently decrease or increase mean SI as compared to control IgG (Appendix 3, table A3.8, complete details of each individual is found in tables A3.9-12). However αIL-12 reduced IFN-γ production in all 3 groups (figure 6.6, see Appendix 3, tables A3.1-4 for details of each donor).

PBMCs from naïve donors had significantly reduced IFN-γ levels when αIL-12 was added to cultures on days 2, 4, and 6 (figure 6.6a, paired $t \geq 2.29$, $p \leq 0.04$, $df = 13$ on all days). By day 8, IFN-γ levels began to increase slightly in αIL-12/PfSE-stimulated cultures, but still remained close to background levels, indicating that IFN-γ production from naive cells is largely IL-12 dependent. Cells from exposed donors also had significantly reduced PfSE-stimulated IFN-γ levels in the presence of αIL-12 on days 2, 4, 6 and 8 (figure 6.6b, paired $t \geq 2.81$, $p \leq 0.01$, $df = 19$). However, αIL-12 did not completely inhibit IFN-γ production and by day 6, IFN-γ levels were still significantly higher than in control IgG/uRBC-stimulated cultures ($t \geq 5.2$, $p < 0.001$, $df = 16$) or in PfSE-stimulated cultures of naive cells ($t \geq 4.56$, $p < 0.001$, $df = 32$), indicating that there is IL-12-dependent and IL-12-independent IFN-γ production from cells of exposed

donors. Although PfSE-stimulated PBMCs from immune donors produced very little IFN-γ, αIL-12 still significantly reduced IFN-γ levels to background levels by day 6 (figure 6.6c, paired $t = 2.18$, $df = 19$, $P = 0.042$).

6.3.3 Production of IL-10 in PfSE-stimulated cell cultures

IL-10 levels were measured in cell supernatants in order to determine whether differences in IL-10 production might explain the differences in IFN-γ responses between groups. IL-10 was measured on days 1, 2, 4, 6 and 8 for naïve and exposed individuals and on days 2, 4, and 6 for immune individuals.
Overall, PfSE-specific IL-10 levels were extremely low in all cultures on all days (table 6.4). IL-10 production was significantly different between uRBC-stimulated and PfSE-stimulated cultures for naïve donors on day 1 (table 6.4a, paired $t = 2.66$, $p > 0.02$, $df = 11$) and for exposed donors on days 4 and 8 (table 6.4b, paired $t \geq 2.68$, $p \leq 0.02$, $df \geq 15$), but levels were still quite low. Almost none of the immune donors had detectable levels of IL-10 (table 6.4c, see Appendix 3, tables A3.13-16 for complete details of each donor). There was also no significant difference between groups in PfSE-specific IL-10 (PfSE-uRBC) levels on all days measured (figure 6.7, Mann Whitney U $W \geq 282$, $p > 0.05$, $df \geq 28$). When individuals were classified as either responders or non-responders for IL-10 production (PfSE > uRBC and > LLD on any day), 16/20 (80%) of exposed donors, 14/18 (78%) of naïve donors and 2/20 (10%) of immune donors were considered responders, ($\chi^2 = 25.21$, $p < 0.001$, $df = 2$). All groups had similar number of responders when looking at IL-10 responses to PHA (18/18 (100%) naives, 19/20 (95%) exposed, 19/20 (95%) immunes). IL-10 production was correlated with neither IL-12 p40 nor IFN-γ responses (data not shown).

6.4 Discussion

The data presented here clearly show that there is an association between the acquisition of clinical immunity to *P. falciparum* infection and *in vitro* downregulation of the pro-inflammatory cytokine, IFN-γ. PBMCs from naïve individuals (who are at high risk of severe malaria if infected) and exposed individuals (who still get sick when infected) produce significant amounts of IFN-γ to PfSE. In contrast, cells from malaria-

immune individuals produce minimal amounts of IFN-γ when stimulated with malaria Ag. IFN-γ production was mainly IL-12-dependent, as shown by decreased IFN-γ levels when neutralising αIL-12 was added to cultures. However, malaria-specific IFN-γ production was not completely neutralised in cultures from exposed individuals indicating that there may have been another source of IFN-γ which was IL-12-independent. There was very little malaria-specific IL-10 produced from cells of all donors, suggesting that this cytokine does not play a major role in regulating IFN-γ production *in vitro*.

6.4.1 The role of IFN-γ in malaria infection

Complete sterilising immunity to malaria rarely, if ever, develops in humans. Rather, there is a state of premunition, where individuals are able to sustain low parasite densities in the absence of clinical symptoms. It is not entirely clear how parasitemia is limited but studies indicate that the release of pro-inflammatory cytokines and Ab-dependent cell-mediated inhibition are involved (37).

However, high levels of circulating pro-inflammatory cytokines are also associated with severe malaria infection (15, 238, 356). Thus, it is not known whether clinical immunity develops as a result of anti-parasitic mechanisms of if the down-regulation of inflammatory cytokines is also involved.

On the basis of observations from *in vitro* cultures it was originally thought that malaria antigens directly stimulated the release of TNF-α, IL-1 and IL-6 from Mø to induce the clinical symptoms (i.e. fever) associated with the inflammatory response (117, 305). But this hypothesis has recently come into question, because it was found that mycoplasma is a strong inducer of TNF-α and can infect long-term malaria cultures (292). This stimulatory mechanism also cannot explain why young infants do not have clinical symptoms upon primary malaria infection (6, 40, 108, 213, 347).

It is now beleived that IFN-γ may be needed to induce the high levels of TNF-α and other inflammatory cytokines which mediate the clinical symptoms of severe malaria infection. Scragg *et al.* have demonstrated that malaria Ag does not induce similar levels of TNF-α and IL-12 as LPS-stimulated PBMCs from naïve individuals *in vitro* (308). Scragg *et al.* also showed that malaria Ag stimulated significant early

IFN-γ production, which is in agreement with the results of this study. Excessive IFN-γ production may explain why naïve individuals or children living in malaria endemic areas (who are primed by either cross-reactive environmental antigens or constant exposure to malaria parasites) are more susceptible to severe malaria than infants (279). In this study, it was demonstrated that cells from clinically immune individuals produced significantly lower levels of IFN-γ to PfSE compared to naïve and semi-immune individuals. These results are in agreement with two other studies in clinically immune Africans (53, 284). Taken together, these results suggest that the downregulation of IFN-γ may therefore be a critical factor in mediating clinical immunity to malaria.

6.4.2 The role of IL-12 in malaria infection

Significant levels of malaria-specific IL-12 were not detected in cell culture supernatants from naïve, exposed or immune individuals by ELISA or RT-PCR (see Chapter 4 results). Although cells from one individual produced as much as 530 pg/ml of IL-12, IL-12 production varied between individuals and was not significantly different between the study populations. However, studies with neutralising αIL-12 demonstrate that IL-12 is produced following PfSE-stimulation, even though the IL-12 may not reach detectable concentrations. The lack of detectable IL-12 could be explained by the fact that minuscule amounts of IL-12 are needed to be biologically active (reasons are discussed in section 4.4), and these very small amounts of IL-12 may bind to IL-12R and are taken out of solution. Additionally, other inflammatory cytokines such as IL-15 and IL-18 (discussed further in Chapter 8) may be upregulated by IL-12, having potent effects (249, 323, 325).

It has been demonstrated in this study that IFN-γ responses are in part regulated by IL-12. PBMCs from naïve individuals produced moderate amounts of IFN-γ which were decreased by the addition of neutralising antibodies to IL-12, suggesting that IFN-γ production is strongly IL-12 dependent. It may be that the primary source of IFN-γ in these individuals is NK cells as a number of studies have demonstrated that NK cell production of IFN-γ to parasitic infections is IL-12 dependent (300, 301, 335). However, αIL-12 only partially blocked IFN-γ production in some naïve individuals suggesting that even in these individuals some IFN-γ production is IL-12 independent. Exposed

individuals still had significantly higher of IFN-γ in cultures with αIL-12 than PfSE-stimulated cultures of naïve individuals. These results suggest that there might possibly be multiple sources of IFN-γ, only one of which is IL-12-dependent. IL-12-independent IFN-γ production in parasitic infections has been shown in other studies (300). In studies by Scragg *et al.* and studies done in our lab, T cells have been found to be one of the sources of early IFN-γ production to malaria Ag in naïve individuals (308)(see Chapter 7). Taken together, these results support the idea that malaria-reactive T cells (primed by other cross-environmental antigens) are involved in the pro-inflammatory response during malaria infection.

6.4.3 Downregulation of the inflammatory response- a role for IL-10?

The minimal amount of IFN-γ produced from cells of immune individuals is not due to T cell anergy; PBMCs from immune individuals proliferated just as strongly to PfSE as cells from naïve and exposed individuals (see Chapter 5). The mechanism by which IFN-γ levels (or the effects of IFN-γ production) are downregulated is not clear. Cells from all individuals produced very little malaria-specific IL-10 and the differences between groups were not significant. The fact that malaria Ag does not induce IL-10 has been confirmed by another recent study (308). Although, IL-10 has been shown to modulate cytokine production to malaria Ag *in vitro* (134), plasma levels of IL-10 are elevated in severe *P. falciparum* infection (15, 135, 154, 263, 356). Additionally IL-10 has been found to stimulate NK cell and T cell IFN-γ production (46, 312). The additional role of IL-10 as a pro-inflammatory cytokine may in part explain why there were significantly more IL-10 responders in the naïve and exposed study populations than in the immune study group. IL-10 thus may not play a major anti-inflammatory role. Further studies need to be done in order to determine whether there are any other cytokines which may downregulate the inflammatory response. A possible cytokine would be TGF-β, as there is known to be an inverse correlation between circulating TGF-β levels and severity of malaria infection in mice (252). The potential role of TGF-β is discussed in Chapter 8.

Table 6.1: Geometric mean IFN-γ (pg/ml) and 95% CI of PfSE-stimulated and uRBC-stimulated PBMCs from a) naïve, b) exposed and c) immune individuals for each day.

a)

	uRBC-stimulated		PfSE-stimulated				
Day	Mean IFN-γ	CI	Mean IFN-γ	CI	t	p	n*
1	61	69, 54	71	85, 59	1.81	0.09	13
2	67	74, 61	92	119, 70	2.54	0.02	19
4	71	79, 63	145	231, 92	3.66	0.002	19
6	70	76, 63	224	371, 136	5.09	< 0.001	19
8	67	73, 61	171	247, 119	5.20	< 0.001	18

b)

	uRBC-stimulated		PfSE-stimulated				
Day	Mean IFN-γ	CI	Mean IFN-γ	CI	t	p	n*
1	46	51, 41	52	61, 45	2.43	0.03	20
2	48	54, 43	89	134, 60	3.46	0.003	20
4	56	68, 45	605	1168, 313	8.46	< 0.001	19
6	79	121, 52	946	1785, 502	9.01	< 0.001	18
8	83	113, 60	1069	1953, 585	9.41	< 0.001	19

c)

	uRBC-stimulated		PfSE-stimulated				
Day	Mean IFN-γ	CI	Mean IFN-γ	CI	t	p	n*
2	57	57.0, 58.0	68	52.9, 87.3	1.42	>0.05	18
4	57	56.7, 57.8	70	59.8, 81.7	2.79	0.02	13
6	58	56.7, 58.6	91	64.3, 127.5	2.75	0.01	20

*there were insufficient cells from some individuals to set up a full set of experiments.

CI- confidence interval.
t- t value for the significance level.
p- p value.
n- number of samples/individuals on given day.

Figure 6.1: Median PfSE-specific (PfSE-uRBC) IFN-γ (pg/ml) production by PBMCs from naïve (■), exposed (▲) and immune (●) donors. Ranges of IFN-γ on all days are 0-2216 pg/ml for naives, -2-4985 pg/ml for exposed and -4-705 pg/ml for immunes.

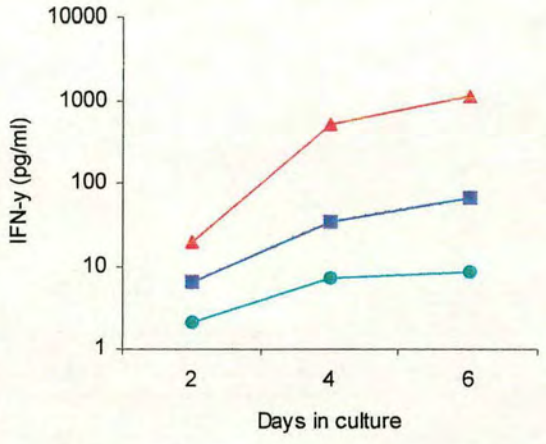


Table 6.2: Number (percentage) of individuals who had positive IFN-γ responses (PfSE ≥ 2XuRBC IFN-γ) on days 2, 4, 6 and total number of responders (positive on any day).

Days	Naives	Exposed	Immunes	χ^2	p
2	3/19 (16%)	5/20 (25%)	1/18 (6%)	2.69	>0.05
4	5/19 (26%)	16/19 (84%)	1/13 (8%)	21.91	<0.001
6	11/19 (58%)	17/18 (94%)	5/20 (25%)	18.74	<0.001
Any day	14/19 (74%)	19/20 (95%)	5/20 (25%)	22.43	<0.001

χ^2- chi-squared value.
p- p value.

Figure 6.2: Kinetic timecourses of lymphoproliferative (■) and IFN-γ (▲) responses to PfSE of PBMCs from a) naïve (n=19), b) exposed (n=20) and c) immune (n=20) individuals (median (PfSE-uRBC) for IFN-γ (pg/ml) and geometric mean SI (PfSE/uRBC) for lymphoproliferation).

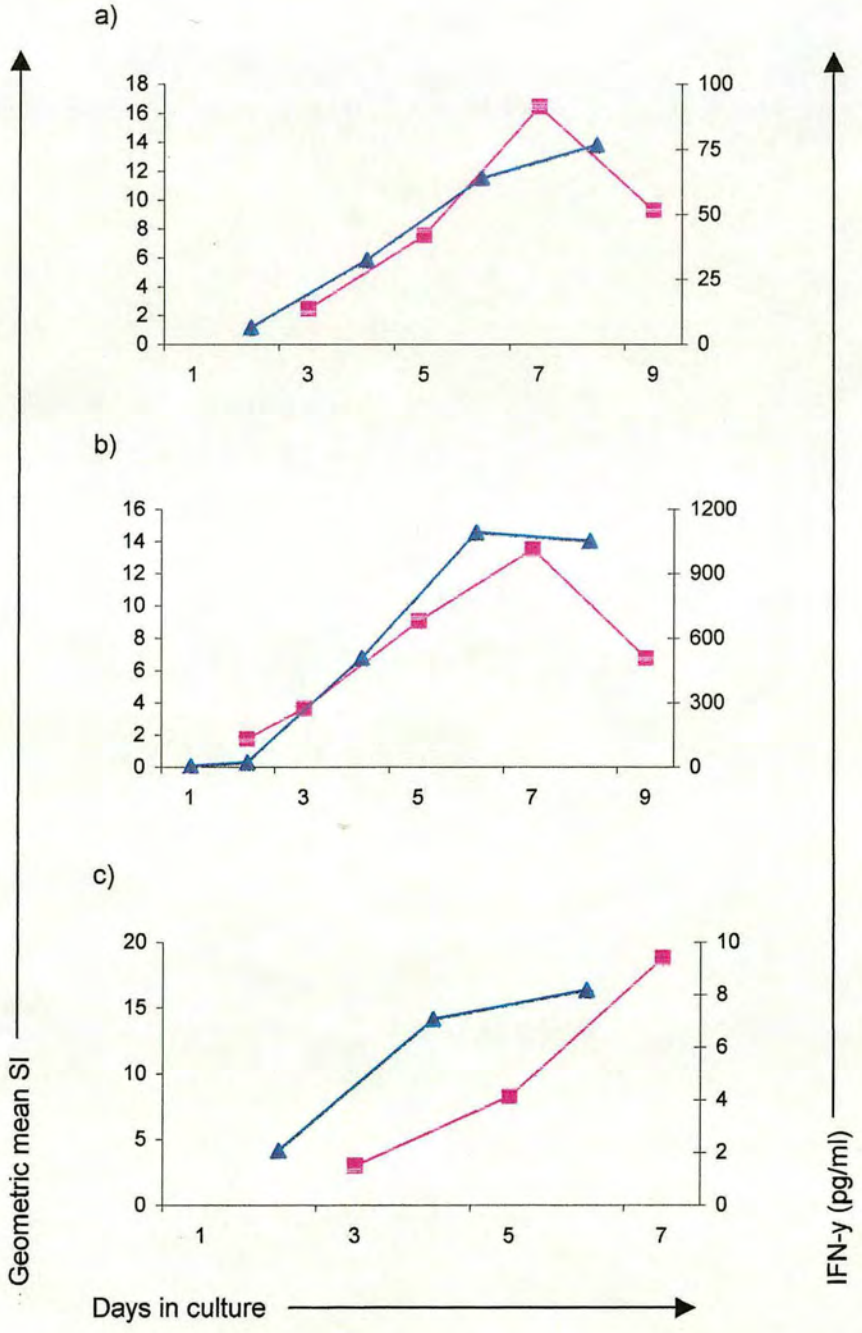


Figure 6.3: Lymphproliferation (SI=PfSE/uRBC) is positively correlated with median IFN-γ production (PfSE-uRBC in pg/ml) for a) naïve, b) exposed and c) immune individuals (values from all the days measured are correlated).

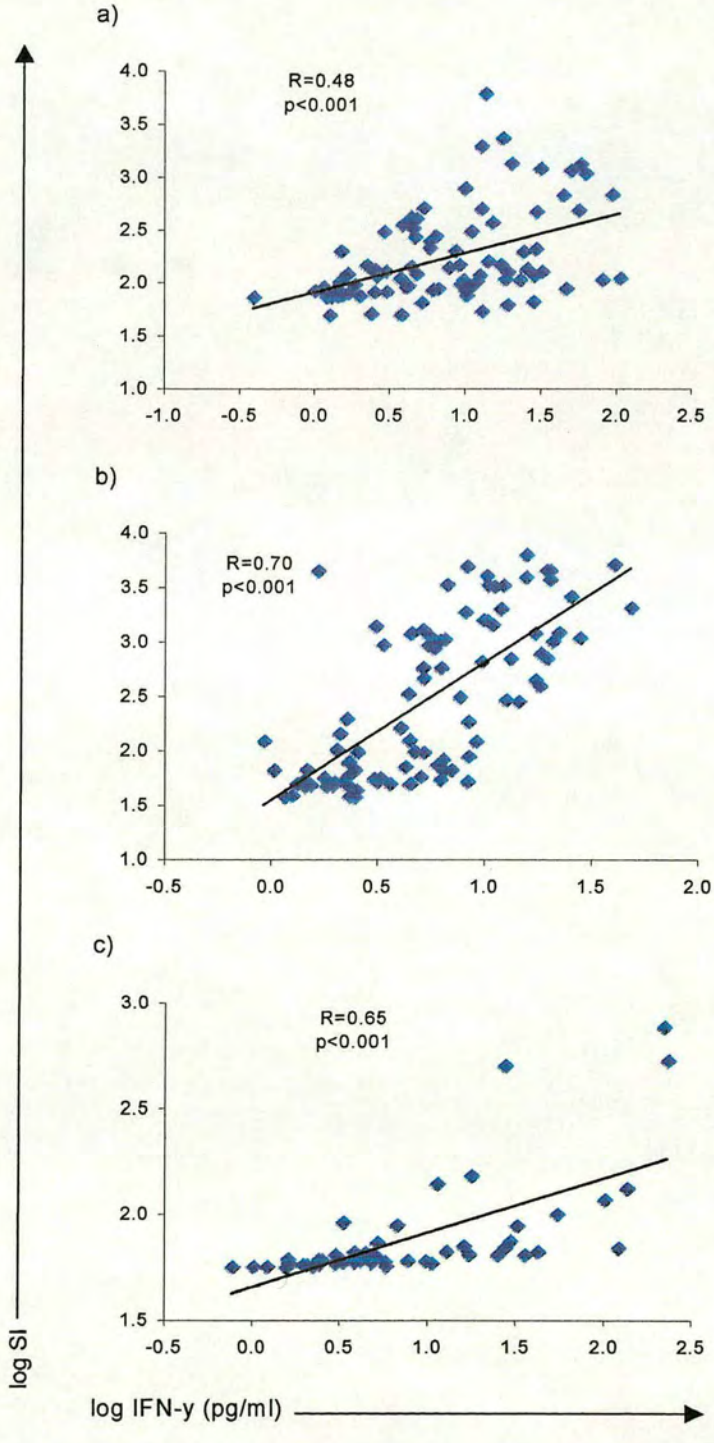


Table 6.3: Geometric mean IL-12 p40 (pg/ml) and 95% CI of PfSE-stimulated and uRBC-stimulated PBMCs from naïve, exposed and immune individuals for day 2.

	uRBC-stimulated		PfSE-stimulated				
Group	**Mean IL-12 p40**	**CI**	**Mean IL-12 p40**	**CI**	**t**	**p**	**n**
Naives	198	176, 223	199	177, 224	0.21	>0.05	18*
Exposed	263	204, 339	279	209, 373	1.93	>0.05	20
Immunes	216	180, 259	217	194, 244	0.06	>0.05	20

* not enough cell culture supernatant was available for 1 donor.

CI- confidence interval.
t- t value for the significance level.
p- p value.
n- number of samples/individuals on given day.

Figure 6.4: PfSE-specific (PfSE-uRBC) IL-12 p40 (pg/ml) production by PBMCs from naïve, exposed and immune donors on day 2. Ranges of IL-12 p40 are -20-28 pg/ml for naives (n=18), -32-530 pg/ml for exposed (n=20) and -545-73 pg/ml for immunes (n=20) (median values denoted). Two extreme values (530 pg/ml and –545 pg/ml) are omitted from this plot.

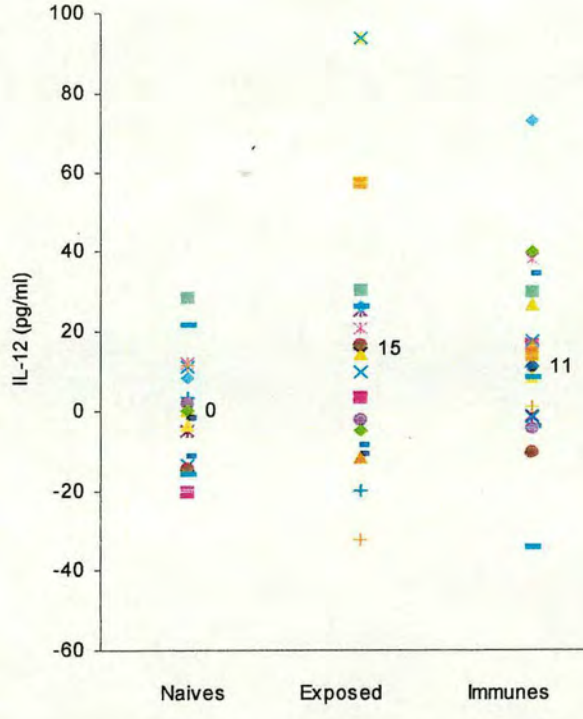


Figure 6.5: Kinetic timecourse of IL-12 p40 (■, PfSE in pg/ml) and IFN-γ (▲, PfSE-uRBC in pg/ml) responses to PfSE from naïve donor N4 (a) and exposed donor E9 (b).

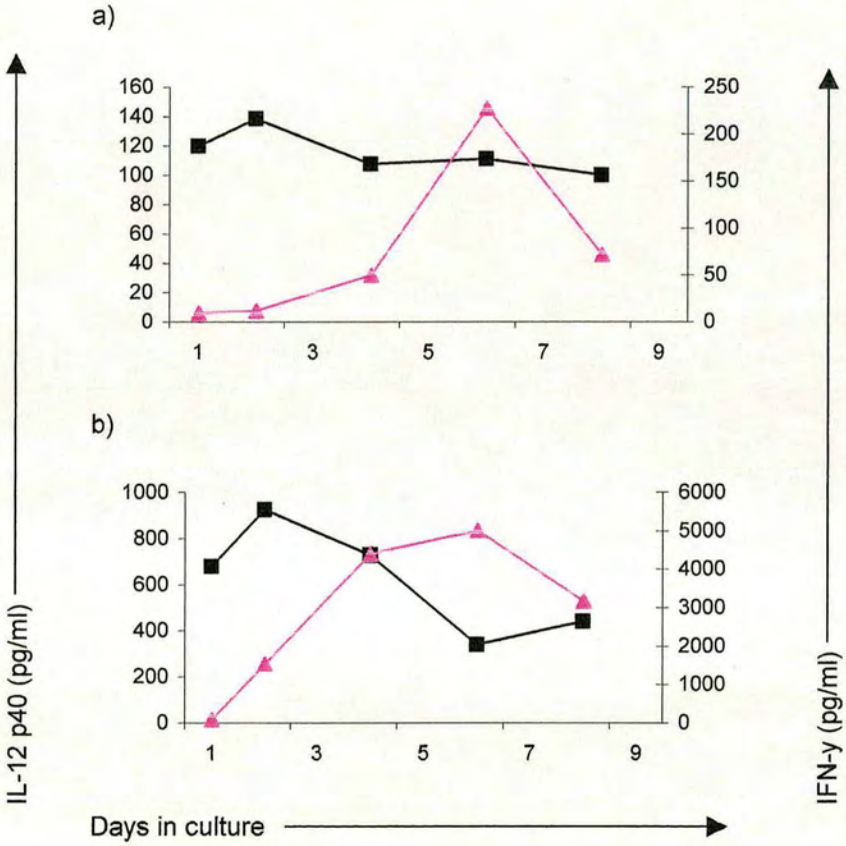


Figure 6.6: Geometric mean IFN-γ (pg/ml) and 95% CI of control IgG/PfSE-stimulated (■) and αIL-12/PfSE-stimulated (▲) PBMCs of a) naïve, b) exposed and c) immune individuals.

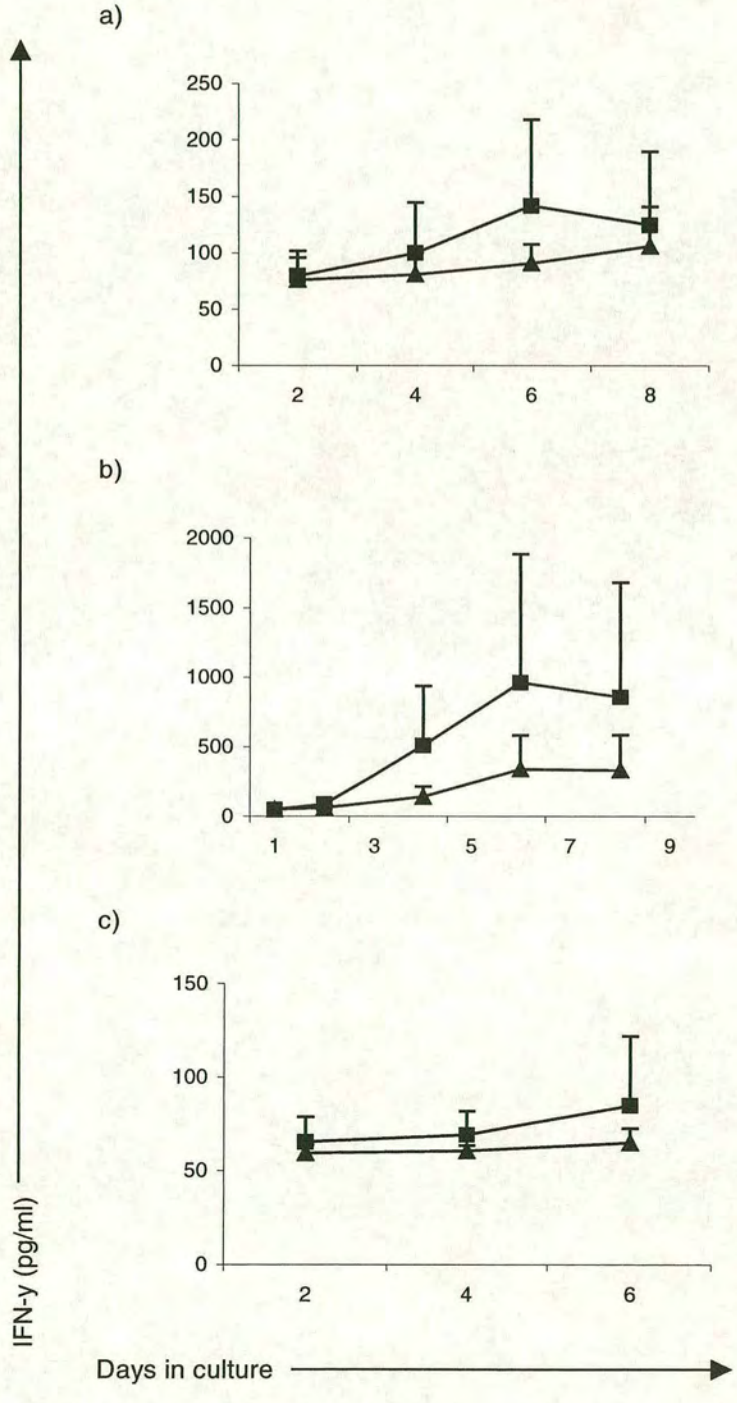


Table 6.4: Geometric mean IL-10 (pg/ml) and 95% CI of PfSE-stimulated and uRBC-stimulated PBMCs from a) naïve, b) exposed and c) immune individuals for each day.

a)

	uRBC-stimulated		PfSE-stimulated				
Day	Mean IL-10	CI	Mean IL-10	CI	t	p	n*
1	50	42, 59	56	45, 69	2.66	0.022	12
2	62	48, 78	62	50, 78	0.49	>0.05	17
4	60	48, 75	61	51, 73	0.48	>0.05	18
6	58	48, 71	61	50, 73	0.71	>0.05	17
8	56	48, 65	59	50, 70	1.18	>0.05	18

b)

	uRBC-stimulated		PfSE-stimulated				
Day	Mean IL-10	CI	Mean IL-10	CI	t	p	n*
1	68	56, 81	67	57, 78	0.59	>0.05	20
2	66	54, 79	67	57, 80	0.37	>0.05	19
4	58	52, 65	63	56, 71	2.68	0.016	17
6	56	49, 63	58	51, 65	0.58	>0.05	17
8	53	47, 59	63	53, 76	3.39	0.004	16

c)

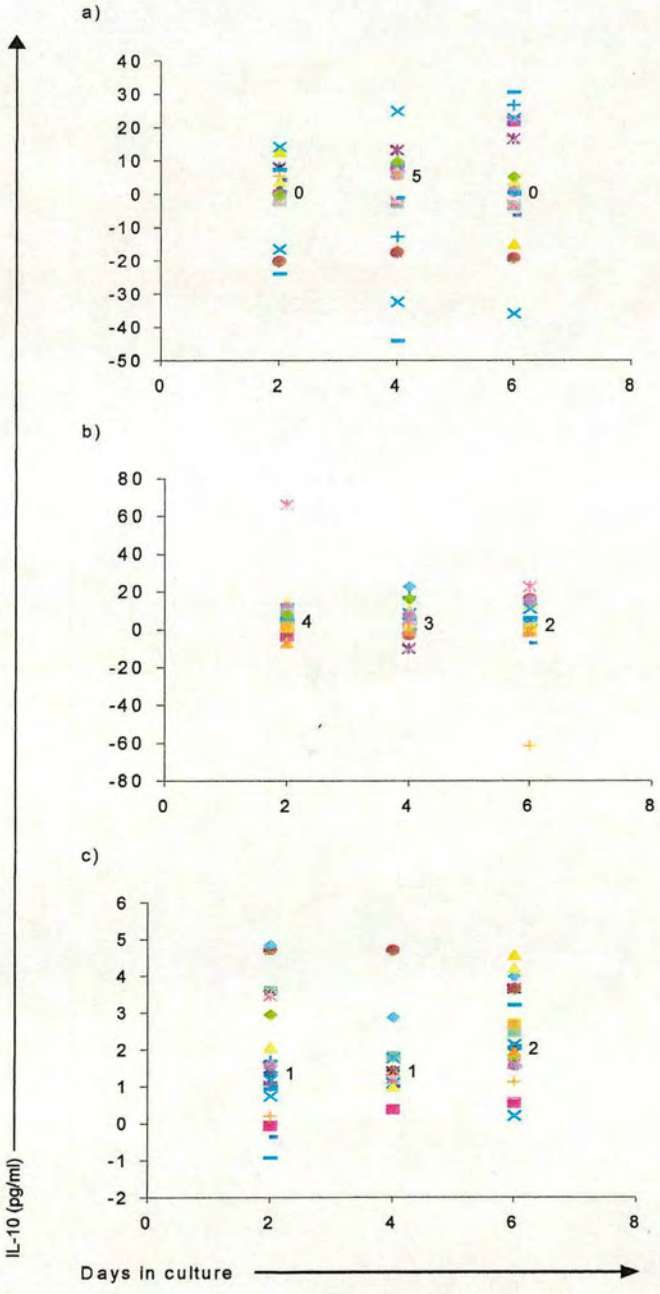
	uRBC-stimulated		PfSE-stimulated				
Day	Mean IL-10	CI	Mean IL-10	CI	t	p	n*
2	42	41, 43	43	42, 45	N/A#	N/A#	18
4	41	40, 42	43	42, 44	N/A	N/A	13
6	41	41, 42	43	42, 44	N/A	N/A	20

* not enough cell culture supernatant was available from some donors.

not applicable because mean IL-10 concentrations were below LLD.

CI- confidence interval.
t- t value for the significance level.
p- p value.
n- number of samples/individuals on given day.

Figure 6.7: PfSE-specific (PfSE-uRBC) IL-10 (pg/ml) production by PBMCs from a) naïve, b) exposed and c) immune donors. Ranges of IL-10 on all days compared (i.e. days 2, 4 and 6) are -44 to 30 pg/ml for naives, -132 to 66 pg/ml for exposed and -0.95 to 4.8 pg/ml for immunes (median values denoted). One extreme values (-132 pg/ml on day 2 for an exposed individual) is omitted from the plot. Numbers of individuals are given in table 6.4.



Chapter 7: Flow cytometric analysis of activated specific lymphocyte subsets and production of IFN-γ in response to *P. falciparum*

7.1 Introduction

There have been numerous studies which have tried to elucidate T cell responses to *P falciparum* infection. However, the results have not necessarily made the T cell immune mechanism any clearer. The controversy lies in the fact that various studies show that different cell subsets are preferentially expanded in response to *P. falciparum* Ag. A number of studies in unsensitised individuals show that CD4+ αβ+ memory cells are stimulated by *P. falciparum* Ag *in vitro* (62, 63, 75, 290), although other studies have demonstrated that γδ+ cells are the principal cell type to respond (20, 115). These results may be due to the fact that different Ag preparations were used (see discussion, section 7.4.2)

In vivo, during acute infections the number of CD4+ αβ+ cells, as well as the numbers of CD8+ and NK cells in peripheral blood are significantly reduced (295, 363), and the percentage and number of γδ+ cells are increased (137, 242, 260, 295). As different cells have different activation requirements, and mediate different effector mechanism, there is a need to determine which cell populations are involved in the response.

In studies of both mouse and human malaria, it has been shown that CD4+, CD8+, γδ+ and NK cells are potent producers of IFN-γ (75, 116, 236, 264, 363). IFN-γ is beneficial in malaria infection, because it is able to inhibit parasite growth (98, 216) and mediate production of other inflammatory cytokines (113). But because there is cellular expansion of cells responding to multiple epitopes in naïve individuals in response to malaria Ag, it is believed that the excessive inflammation caused by these cells may contribute to pathology during infection. Although these cells are important players in cell mediated immunity, whether or not they are beneficial depends on the timing, location and strength of the response.

The cell subsets which expand in response to malaria Ag *in vitro* have been isolated from individuals who have never been exposed to malaria or in individuals acutely infected with *P. falciparum*. Less is known about the differences in cellular responses between naïve individuals and healthy individuals living in malaria endemic

areas. Flow cytometry analysis combined with intracellular cytokine staining (ICS) are powerful tools that show which cells are being activated (i.e. blasting) and which cells are producing cytokines. Here I present a study, which uses these experimental assays, comparing phenotypes of activated cells responding to *P. falciparum* Ag *in vitro* from naïve, exposed and clinically immune individuals. I also present preliminary data on cellular function by examining which cell subsets produce IFN-γ in response to malaria Ag.

7.2 Materials and Methods

7.2.1 Study population and cell culture

Ten to 30 ml of venous blood was taken from 13 naïve individuals, 19 exposed individuals and 18 immune subjects and processed for *in vitro* culture (see section 2.1.1 for description of study populations and section 2.4 for method of cell separation). Blood samples from 3 additional naïve individuals were used as controls when setting up cultures in Ghana. PBMCs from 3 naïve donors were used for ICS, and cells from the rest of the donors were used for cell surface staining and analysis by flow cytometry. Cells were plated at 10^6-$2x10^6$/well in flat-bottomed 24-well plates in a total of 1 or 2 ml complete culture medium. For control or mitogen/antigen culture, $2\text{-}4x10^6$ cells were available for cell surface staining (except for immune individuals where a limited number of cells, usually $<10^6$, was available); see Appendix 4, tables A4.1-4 for cell counts. Six $x10^6$ cells were used for ICS.

For cell surface staining, cells were cultured for 7 days with PfSE, PHA (i.e. positive mitogen control) or PPD (i.e. classical recall T cell Ag). Culture medium alone and uRBC were used as negative controls (concentrations of stimuli are given in sections 2.2.3 and 2.3). Because of the limited amount of blood taken, cells from immune donors were stimulated only with uRBC or PfSE. Additionally, PBMCs from naïve and immune donors were cultured for 3 days with uRBC or PfSE. For ICS, cells were cultured for 7 days with uRBC or PfSE. Culture medium alone was used as a negative control, and SEB was used as a positive control for intracellular IFN-γ staining.

7.2.2 Cell surface staining

After 0, 3 or 7 days, PBMCs were harvested into 15 ml conical tubes and washed twice with PBS supplemented with 0.1% NaN_3 and 0.1% BSA (FACs PBS). Cells were prepared for 3-colour flow cytometry by resuspending in FACs PBS at $2x10^6$/ml and distributed equally into round-bottomed 96-well microtitre plates (i.e. up to $2x10^5$/well). Twenty microliters of diluted antibody were added to each well and PBMCs were incubated at 4 °C for 40 minutes (see tables 7.1 and 7.2 for staining combinations and cell subsets identified by each stain, Ab concentrations are detailed in section 2.8.2). Cells were washed twice and resuspended in 300 µl of FACs PBS for phenotypic analysis.

7.2.3 Intracellular cytokine staining

The ICS procedure is based on the original protocol developed by Jung *et al.* (161). Eighteen hours before cells were harvested, 3 µg/ml of Brefeldin A was added to cultures for protein transport inhibition, in order to increase the IFN-γ signal. PBMCs were harvested, washed twice with PBS and resuspended at a final concentration of 10^6/ml in PBS supplemented with 1% fetal calf's serum and 0.1% NaN_3 (staining buffer, buffers are detailed in section 2.9.1). Cells were surface stained as described in section 7.2.2, and then washed once with PBS. PBMCs were fixed for 15 minutes at RT in the dark, washed and then stained with FITC- or PE-labelled anti-IFN-γ and permeabilised for 30 minutes at 4 °C in the dark. Cells were washed a final time and then resuspended in 0.5 ml PBS for FACS analysis. Complete details of the ICS protocol are given in section 2.9, and staining combinations are given in table 7.3.

7.2.4 Data analysis

Cell phenotyping was done by flow cytometry and analysed by Cell Quest software package (see section 2.8.3). RBCs, dead cells and apoptosing cells were gated out and 10,000 to 20,000 events were acquired for each staining combination. Resting cells (R1) and lymphoblasts (R2) were gated separately, detected by FSC versus SSC, and analysed for the different staining combinations. Gating was based on unstained controls and samples single-stained with FITC-, PE- or TRI-conjugated antibodies. One cell aliquot was used to determine cell viability by trypan blue

exclusion and absolute cell numbers harvested from each stimulated or unstimulated culture. Percentages and absolute numbers of cell subsets were calculated excluding dead cells. Comparisons within each group were assessed by paired t test and between groups by student's t test on arithmetic mean percentages and on log transformed absolute cell counts.

7.3 Results

7.3.1 Phenotypic analysis of PBMCs cultured with PfSE; T cells and NK cells

Cells were stained for CD3 (T cells), CD20 (B cells) and CD56 (NK cells) on days 0, 3 (naïve and immune donors only) and 7, and percentages and absolute cell counts were compared between uRBC- and PfSE-stimulated cells for each group (tables 7.4-7.7, day 0 data not given). Summary data of all subsets for each study population are given in Appendix 4, tables A4.5-4.10, and cell surface staining for PBMCs of each donor are given in Appendix 4, tables A4.11-4.18. There was no consistent difference in the numbers of cells retrieved or the total number of viable cells from uRBC- or PfSE-stimulated cultures (data not shown). However, there were significant increases in the proportion and in the absolute numbers of blast cells (R2) in PfSE-stimulated cultures compared to uRBC-stimulated cultures of naïve (paired $t \geq 2.44$, $p \leq 0.04$, $df = 9$) and exposed (paired $t \geq 7.53$, $p \leq 0.001$, $df = 17$), but not of immune (paired $t \leq 1.98$, $p > 0.05$, $df = 17$) donors for each day (tables 7.4-7.7). Representative dot plots for PfSE- and uRBC-stimulated PBMCs of naïve and exposed individuals are shown in figure 7.4b-c. Interestingly, naïve donors had a significantly higher proportion and number of lymphoblasts on day 7 compared to both exposed (student's $t \geq 3.38$, $p \leq 0.002$, $df \geq 26$) and immune individuals (student's $t \geq 5.67$, $p \leq 5.67$, $df \geq 26$, figure 7.1), possibly indicating greater activation of T and/or B cells in response to PfSE in naïve donors.

For all study populations, blasting cells in PfSE cultures were predominantly T cells on day 3 (data not shown) and on day 7 (figure 7.2a). Exposed donors had a significantly lower proportion of T cells (mean = 57.5%) compared to naïve

(mean = 83.4%) and immune (mean = 73.7%) populations (student's $t \geq 3.03$, $p \leq 0.005$, $df \geq 26$). However, there was a higher proportion of blasting B cells and NK cells in PfSE-stimulated cultures of exposed individuals which compensated for the smaller proportion of T cells (figure 7.2b-c). Naïve donors had a significantly higher absolute number of NK cells in PfSE cultures than in uRBC cultures on day 3 (table 7.5a) and day 7 (table 7.7a, statistics are in the tables). For malaria-exposed donors, the number of NK cell blasts in PfSE-stimulated cultures were also significantly higher than in uRBC-stimulated cultures (table 7.7.b) and were significantly higher than for naïve donors on day 7 (student's $t = 2.38$, $p = 0.03$, $df = 25$). It should be noted that exposed individuals also had a significantly higher proportion of NK cell blasts in uRBC cultures compared to naïve individuals (student's $t = 5.37$, $p < 0.001$ $df = 25$), indicating a non-specific activation of NK cells by uRBC in exposed individuals. In contrast, immune donors had very few malaria-specific NK cell blasts (tables 7.5c and 7.7c). The number of NK cell blasts were lower in immunes than in both naïve and exposed donors, although the difference between naïve and immune donors was not significant (student's $t = 1.43$, $p = 0.17$, $df = 24$).

7.3.2 αβ+ and γδ+ TcR usage

Overall, TcR αβ+ cells were the predominant lymphoblasts in naïve donors (tables 7.8a and 7.9a, 53% of total blasting PBMCs). The percentage and absolute numbers of TcR αβ+ blast cells were significantly higher in PfSE-stimulated cultures than in uRBC-stimulated cultures (paired $t \geq 9.95$, $p \leq 0.001$, $df = 7$). Although TcR αβ+ cells were measured in cell cultures of exposed individuals, the staining did not work (data not shown). However, for both naïve and exposed populations, TcR γδ+ cells were a small percentage of the total blasting cells (table 7.8, range 9-15% for naive, 2-13% for exposed individuals). Although, TcR γδ+ cells were such a small proportion of total blast cells, both naïve and exposed populations had significantly higher numbers of TcR γδ+ cells in PfSE cultures versus uRBC cultures (tables 7.8b and 7.9b, paired $t \geq 3.01$, $p \leq 0.008$, $df \geq 9$). Additionally, both the percentage and absolute number of TcR γδ+ cells in PfSE-stimulated cultures was significantly higher in naïve than exposed donors (student's $t \geq 4.69$, $p < 0.001$, $df = 24$).

7.3.3 CD45RA+ and CD45RO+ cells

The absolute number and percentage of T cells which were CD45RO+ were consistently higher in PfSE cultures than uRBC cultures for all naïve and exposed donors (see Appendix 4, tables A4.11-A4.14, paired $t \geq 4.77$, $p < 0.001$, $df = 9$). CD45RO+ T cells were higher in proportion and number than CD45RA+ T cells in PfSE-stimulated cultures for both for naïve and exposed individuals (tables 7.8 and 7.9). An increase in CD45RO+ cells was matched by an apparent decrease in CD45RA+ cells and vice versa in uRBC and PfSE cultures. Although, the percentage of CD45RA+ T cells tended to be higher in uRBC-stimulated cultures than in PfSE-stimulated cultures, there was no consistent difference in the absolute numbers of CD45RA+ T cells for the 2 stimuli (see Appendix 4, tables A4.12 and A4.14). The number of CD45RA+ and CD45RO+ T cells were significantly higher in PfSE-stimulated cultures of naïve individuals than of exposed individuals (student's $t \geq 4.07$, $p < 0.001$, $df = 26$).

7.3.4 CD4+/CD8+ ratios

For both naïve and exposed populations, absolute numbers of CD4+ and CD8+ lymphoblasts were significantly higher in PfSE-stimulated cultures than in uRBC-stimulated cultures (tables 7.8b and 7.9b). For naïve donors, the ratio of CD4+/CD8+ lymphoblasts in PfSE-stimulated cultures was approximately 40:1. In contrast, in exposed donors the CD4+/CD8+ ratio was approximately 1.7:1. This was due both to a fall in the percentage and number of CD4+ blasts and a rise in the percentage and number of CD8+ blasts in exposed donors (figure 7.3).

7.3.5 IFN-γ production to PfSE from NK, $\alpha\beta$+ and $\gamma\delta$+ cells

In order to determine the cellular source of IFN-γ in PfSE-stimulated cultures, PBMCs from 3 naïve donors were stimulated with uRBC or PfSE, labelled with monoclonal antibodies (table 7.3) and analysed by flow cytometry (see Appendix 4,

tables A4.19-20 for details of each individual). The majority of IFN-γ+ cells were in the blast population, and a higher proportion was found in PfSE-stimulated than uRBC-stimulated cultures (figure 7.4, mean 4.7 ± 1.8% for PfSE, 1.6 ± 0.5% for uRBC). As can be seen from tables 7.5a and 7.5b, the major proportion of PfSE-stimulated cells in the lymphoblast population are T cells. TcR αβ+ cells represent the dominant proportion of T cells, with 60-80% of these staining positive for IFN-γ. There is a greater than 3-fold increase in the percentage of CD56+ IFN-γ-staining cells and a greater than 2-fold increase in the percentage of TcR γδ+ IFN-γ-staining cells in PfSE versus uRBC cultures. Eighty-five to 100% of CD56+ cells and TcR γδ+ T cells produced IFN-γ in the blast population. Hence, a mixture of cells produce IFN-γ in response to PfSE.

7.4 Discussion

In order to determine if there are differences in cellular proliferation/activation during *P. falciparum* infection, the *in vitro* responses to PfSE of cells from malaria-naïve, malaria-exposed and clinically immune individuals were compared. An attempt was also made to examine which of these cells produce IFN-γ. It was not possible to examine differences in all cellular subsets, particularly in immune donors, however some initial conclusions can be made from this data. First, for naïve donors, PfSE-stimulated cells were predominantly CD4+, TcR αβ+, CD45RO+ T cells, but significant numbers of CD8+, CD56+ and TcR γδ+ cells were also stimulated. TcR αβ+ TcR γδ+ and NK cells all produced IFN-γ in PfSE cultures of naïve individuals. Although, cells from naïve donors had much higher numbers of activated cells than exposed and immune donors, the phenotype of responding cells in exposed individuals is similar to that in naïve individuals (i.e. predominant CD4+, CD45RO+ T cells, TcR αβ+ usage not known). However, exposed donors had much higher percentages of CD8+ T cell blasts and B lymphoblasts than naïve individuals. Finally, immune individuals had much lower numbers of activated cells, with no significant differences in cell subsets between uRBC- and PfSE stimulated cultures.

7.4.1 Immune response in naïve donors

A number of studies have demonstrated that cells from individuals who have never been exposed to malaria can respond to malaria antigens *in vitro* (20, 62, 63, 75, 115, 290, 370). It is generally agreed that the cellular response to malaria antigens is polyclonal (159) and is a result of cross-reactive responses of cells primed by other environmental antigens (62, 63, 97). However, it is not known whether this cross-reactive response is beneficial or harmful. Naïve donors, who are at greater risk of severe clinical illness if infected with malaria, had higher absolute numbers of blasting cells than either exposed or immune donors. Numbers of CD3+, CD4+, CD20+, CD45RA+, CD45RO+, and TcR γδ+ lymphoblasts were higher in PfSE-stimulated cultures of naïve individuals than exposed individuals. However, it should be noted that in both exposed and immune populations, there was a non-specific response to uRBC, possibly indicating sensitisation to RBC antigens following repeated bouts of malaria.

There are a number of explanations for the observed differences in cell numbers between these groups. First, there could have been variations in experimental set-up as samples of each group for flow cytometry were run at different times and locations. Lymphoblasts (R2) were identified on the basis of their FSC and SSC characteristics. Compensation parameters for FSC versus SSC were not identical for samples of immune donors versus naïve and exposed donors. As a result, red blood cells and dead cells were a slightly larger proportion of the total numbers of cells (data not shown). Second, a decrease in circulating malaria-reactive cells could be a result of sequestration in organs where cells are needed to fight off infection (146, 363), as 8/18 immune donors were sub-clinically infected with malaria. However, neither of these reasons explains why exposed individuals had lower numbers of cells than naïve donors, as compensation parameters were identical to naïve donors and only 1 individual was sub-clinically infected. The most likely reason is that malaria Ag polyclonally activates a larger proportion of cells in naïve individuals, and that this response is downregulated in exposed and immune individuals by acquired immune responses. The data indicate that immune responses in individuals living in malaria-endemic areas are specific, as can be seen from studies which demonstrate that only cells from malaria-exposed individuals respond to purified malaria Ag, while both cells

from both naïve and exposed individuals respond to crude malaria Ag (284). Thus, higher numbers of PfSE-stimulated cells in naïve individuals may reflect an excessive activation of cross-reactive cells which may lead to pathogenic infection. In other words, the polyclonal, cross-reactive T cell response in naïve donors may be downregulated in exposed and immune donors to be replaced by malaria-specific T cell responses.

7.4.2 αβ+ and γδ+ T cells in malaria infection

PfSE-stimulated T cells of naïve donors were predominantly TcR αβ+ (> 50%). Although αβ staining of cells from exposed individuals did not work due to a technical problem with the Ab, it could be concluded from the small proportion of TcR γδ+ cells that the predominant cells from exposed individuals were also TcR αβ+. This data is confirmed by previous work in our lab which demonstrated that PfSE-stimulated T lymphoblasts of clinically immune adult Gambians are predominantly αβ+ (Riley *et al.*, unpublished data).

These results are in agreement to a number of studies (63, 75) but seem to contradict others which state that there is a preferential expansion of γδ+ T cells (20, 112, 115). However, Waterfall *et al.* 1998 showed that using live rather than killed parasite, or adding exogenous IL-2, increased γδ+ T cells in culture (354). Additionally, a number of studies have demonstrated γδ+ cellular proliferation is dependent upon αβ+ cells, which are potent producers of IL-2 (84, 291, 319).

Although αβ+ T cells are the major population of cells responding to malaria Ag, there was a significant expansion of γδ+ T cells in PfSE-stimulated when compared with uRBC-stimulated cultures for both malaria-naïve and malaria-exposed adults. γδ+ T cells may interact with malaria Ag through an alternative pathway to αβ+ T cells, as they are able to bind non-protein antigens (294). Elloso *et al.* showed that γδ+ T cells inhibit parasite replication by coming into direct contact with merozoites (85). However, naïve individuals had significantly higher numbers of malaria-specific γδ+ T cells than exposed individuals. Elevated circulating levels of γδ+ T cells have been found in individuals with acute *P. falciparum* infection (51, 137, 145). In contrast, there was no increase in γδ+ T

cells found in children living in malaria-endemic areas regardless of severity of disease (145). Taken together, these results suggests that γδ+ T cells may play a pathogenic role in malaria infection and may be downregulated during acquisition of clinical immunity.

7.4.3 PfSE stimulates both CD45RA+ and CD45RO+ cells

The majority of PfSE-responding cells in both naïve and exposed adults were CD45RO+, which is in agreement with other studies on cells of naïve individuals (75, 159). These results may indicate that the majority of cells which are responding have been primed previously either by common environmental antigens or by malaria antigens. However CD45RA+ cells of both naïve and exposed individuals also responded to PfSE. These results, and other studies, indicate that naïve cells can also respond to malaria antigens (97). However, CD45RA+ and CD45RO+ expression does not necessarily indicate naïve versus memory cells. Rather, these cell surface markers may reflect inactivated versus activated cells. Hamann *et al.* 1996 demonstrated that cells which are CD45RO+ can revert back to CD45RA+ expression in the absence of constant antigenic stimulation (125).

7.4.4 CD4+ and CD8+ T cells in malaria infection

A number of studies have demonstrated that CD4+ T cells are the primary cells responding to PfSE *in vitro* in previously unexposed individuals (62, 63, 290, 370). PfSE-stimulated PBMCs of naïve individuals were also predominantly CD4+ in this study, and cell numbers responding were significantly higher than in exposed individuals. Interestingly, exposed individuals also showed activation of CD8+ cells that was not seen in naïve individuals. Expansion of CD8+ cells thus appears to be a malaria specific response. These results may indicate that CD4+ cells may help to exacerbate infection if in excess and that responses mediated by CD8+ cells may be beneficial in clearing infection. Studies in mice have shown that immunity to pre-erythrocytic liver-stage malaria is dependent upon CD8+ T cells (78). No such studies

are reported for responses mediated by CD8+ cells in immunity to blood stage infections.

7.4.5 Cellular function of NK, CD4+, CD8+, and γδ+ cells in malaria infection

Phenotypic analysis is important, but does not demonstrate the function of each subset. ICS was done on PfSE-stimulated and uRBC-stimulated PBMCs of 3 naïve individuals in order to determine which cells were producing IFN-γ. TcR αβ+ cells were the main producers of IFN-γ, but significant proportions of TcR γδ+ and NK cells also produced IFN-γ to PfSE. These data correlate well with previous studies which demonstrated that both CD4+ αβ+ cells and γδ+ cells are potent producers of IFN-γ during malaria infection (75, 116, 264). Although NK cell production of IFN-γ has not been previously reported in human malaria infection, IFN-γ production by NK cells is involved in resistance to murine *P. c. chabaudi* AS infection (236).

Although further studies need to be done in order to determine if there are any differences between naïve, exposed and immune individuals as to which cells produce IFN-γ, some tentative conclusions can be made based on these results and the results from Chapter 6. PfSE-stimulated cells of exposed individuals produced significantly higher levels of IFN-γ than cells of naïve or immune individuals. A possible source of this IFN-γ is CD8+ cells. Elevated circulating levels of CD8+, IFN-γ+ cells were found in convalescent individuals during drug-induced clearance of *P. falciparum* infection (363). IFN-γ could also be produced by NK cells in exposed individuals, as they had significantly higher proportions of NK cells than naïve individuals. The hypothesis that NK cells are a major source of IFN-γ in malaria infection is further supported by the fact that cells from immune individuals produced minimal amounts of IFN-γ and that immune individuals had almost no PfSE-stimulated NK cells. Finally, because exposed individuals produce higher levels of IFN-γ but have a lower risk of severe malaria than naïve individuals, there must be cells which produce anti-inflammatory cytokines which minimise the effects of IFN-γ. Exposed and immune individuals had higher percentages of responding B cells and higher ratios of B lymphocytes to T lymphocytes than naïve individuals (table 7.6). B lymphocytes have been shown to provide signals to CD4+ cells to produce anti-inflammatory cytokines during *P. c. chabaudi* infection (196).

Taken together, these results suggest B cells may be involved in the anti-inflammatory response during malaria infection. Further studies are required to determine the cellular sources of downregulatory cytokines in *P. falciparum* infection (discussed further in Chapter 8).

Table 7.1: Ab staining combinations for cell surface markers used in flow cytometric analysis for a) naïve, b) exposed and c) immune individuals (concentrations of each Ab are given in section 2.8.2).

a)

FITC-labelled	PE-labelled	TRI-labelled
CD20	CD56	CD3
CD45RA	CD45RO	CD3
TcR αβ	CD4	CD3
TcR γδ	CD8	CD3

b)

FITC-labelled	PE-labelled	TRI-labelled
CD20	CD56	CD45RO
CD45RO	CD45RA	CD3
TcR αβ	CD45RO	CD3
TcR γδ	CD45RO	CD3
CD4	CD45RO	CD3
CD8	CD45RO	CD3
Vγ9	CD45RO	CD3
Vδ1	CD45RO	CD3

c)

FITC-labelled	PE-labelled	TRI-labelled
CD20	CD56	CD3

Table 7.2: Cellular subsets which are detected by the antibodies to the cell surface markers.

Cell surface marker	Cell Type
CD20	B lymphocytes
CD56	NK cells
CD3	T lymphocytes
CD4	Helper T lymphocytes
CD8	Cytotoxic T lymphocytes
CD45RA	Naïve cells
CD45RO	Activated (memory) cells
TcR αβ	T cell receptor containing α and β chains
TcR γδ	T cell receptor containing γ and δ chains
Vγ9	T cell receptor containing γ9 chain
Vδ1	T cell receptor containing δ1 chain

Table 7.3: Three-colour staining combinations used for ICS.

FITC	PE	TRI
IFN-γ	CD56	CD3
TcR αβ	IFN-γ	CD3
TcR γδ	IFN-γ	CD3

Table 7.4: Percentages of lymphoblasts (R2), and percentages of lymphoblasts that are T lymphocytes (CD3+), B lymphocytes (CD20+), or NK cells (CD56+) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve (n = 10) and b) immune (n = 16) donors for **day 3** (arithmetic mean and SEM indicated).

a) naïves

	uRBC-stimulated		PfSE-stimulated			
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**t**	**p**
R2	1.49	0.09	1.91	0.15	2.44	0.04
CD3+	81.5	2.3	72.3	2.4	4.88	0.001
CD20+	2.7	0.6	3.6	0.8	1.29	>0.05
CD56+	0.2	0.2	2.2	0.6	3.59	0.006

b) immunes

	uRBC-stimulated		PfSE-stimulated			
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**t**	**p**
R2	1.92	0.30	2.33	0.43	1.98	>0.05
CD3+	70.6	2.3	70.7	2.6	0.06	>0.05
CD20+	19.9	1.5	10.0	1.5	0.52	>0.05
CD56+	3.6	0.7	2.7	0.6	0.96	>0.05

SEM- Standard error of the mean.

t- t value for the significance level.

p- p value.

Table 7.5: Total numbers of lymphoblasts (R2), and total numbers of lymphoblasts that were T lymphocytes (CD3+), B lymphocytes (CD20+), or NK cells (CD56+) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve (n = 10) and b) immune (n = 16) donors for **day 3** (geometric mean and 95% CI indicated, cell count is per 10^6 cell plated out on day 0).

a) naïves

	uRBC-stimulated			PfSE-stimulated				
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**t**	**p**
R2	8165	6820	9776	12108	10151	14443	5.00	0.001
CD3+	6634	5398	8153	8643	6963	10728	3.54	0.006
CD20+	183	111	301	343	207	566	3.36	0.008
CD56+	2	1	9	95	16	552	2.51	0.033

b) immunes

	uRBC-stimulated			PfSE-stimulated				
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**t**	**p**
R2	4039	1897	8603	3286	1541	7009	0.87	>0.05
CD3+	2823	1263	6308	2288	1023	5118	0.87	>0.05
CD20+	331	105	1040	169	38	746	1.40	>0.05
CD56+	66	18	241	37	8	167	1.23	>0.05

CI- Confidence interval.

t- t value for the significance level.

p- p value.

Table 7.6: Percentages of lymphoblasts (R2), and percentages of lymphoblasts that are T lymphocytes (CD3+), B lymphocytes (CD20+), or NK cells (CD56+) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve (n = 10), b) exposed (n = 18) and c) immune (n = 18) donors for **day 7** (arithmetic mean and SEM indicated).

a) naives

	uRBC-stimulated		PfSE-stimulated			
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**t**	**p**
R2	0.4	0.1	8.0	1.0	7.53	<0.001
CD3+	62.8	5.1	83.4	2.8	4.93	0.001
CD20+	14.6	3.2	7.7	1.6	2.77	0.020
*CD56+	3.7	1.7	2.5	0.7	0.65	>0.05

b) exposed

	uRBC-stimulated		PfSE-stimulated			
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**t**	**p**
R2	3.3	0.4	4.6	0.5	3.90	0.001
CD3+	53.4	4.2	57.5	4.1	2.20	0.042
CD20+	10.1	1.6	13.7	1.9	2.45	0.030
CD56+	8.3	1.6	12.2	1.6	2.28	0.035

c) immunes

	uRBC-stimulated		PfSE-stimulated			
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**t**	**p**
R2	1.2	0.3	1.8	0.4	1.43	>0.05
CD3+	68.6	3.9	73.7	2.7	1.55	>0.05
CD20+	10.3	1.8	9.6	1.8	0.53	>0.05
#CD56+	4.8	1.3	4.7	0.7	0.07	>0.05

* n = 9

n = 17

SEM- Standard error of the mean.

t- t value for the significance level.

p- p value.

Table 7.7: Total numbers of lymphoblasts (R2), and total numbers of lymphoblasts that were T lymphocytes (CD3+), B lymphocytes (CD20+), or NK cells (CD56+) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve(n = 10), b) exposed (n = 18) and c) immune (n = 18) donors for **day 7** (geometric mean and 95% CI indicated, cell count is per 10^6 cell plated out on day 0).

a) naives

	uRBC-stimulated			PfSE-stimulated				
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**t**	**p**
R2	1441	576	3605	41120	26464	63892	11.12	<0.001
CD3+	880	326	2376	34131	20610	56522	11.30	<0.001
CD20+	121	513	29	2597	4151	1625	5.71	<0.001
*CD56+	14	2	103	374	65	2156	3.58	0.007

b) exposed

	uRBC-stimulated			PfSE-stimulated				
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**t**	**p**
R2	9928	6961	14160	14593	11279	18881	2.91	0.010
CD3+	4850	3652	6441	7706	5825	10193	3.35	0.004
CD20+	800	500	1200	1700	1200	2500	4.97	<0.001
CD56+	560	338	927	1464	1044	2054	4.00	0.001

c) immunes

	uRBC-stimulated			PfSE-stimulated				
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**t**	**p**
R2	1787	929	3439	2712	1329	5535	1.54	>0.05
CD3+	1189	587	2408	1977	942	4151	1.61	>0.05
CD20+	93	28	316	128	36	460	0.57	>0.05
#CD56+	54	24	124	108	38	306	1.55	>0.05

* n = 9

n = 17

CI- Confidence interval.

t- t value for the significance level.

p- p value.

Figure 7.1: Comparison of total numbers of cells in the lymphoblast (R2) population of uRBC-stimulated (left hand columns) and PfSE-stimulated (right-hand columns) cultures between naïve, exposed and immune donors (geometric mean indicated). Numbers are expressed as number of blast cells harvested on **day 7** per 10^6 cells plated out on day 0.

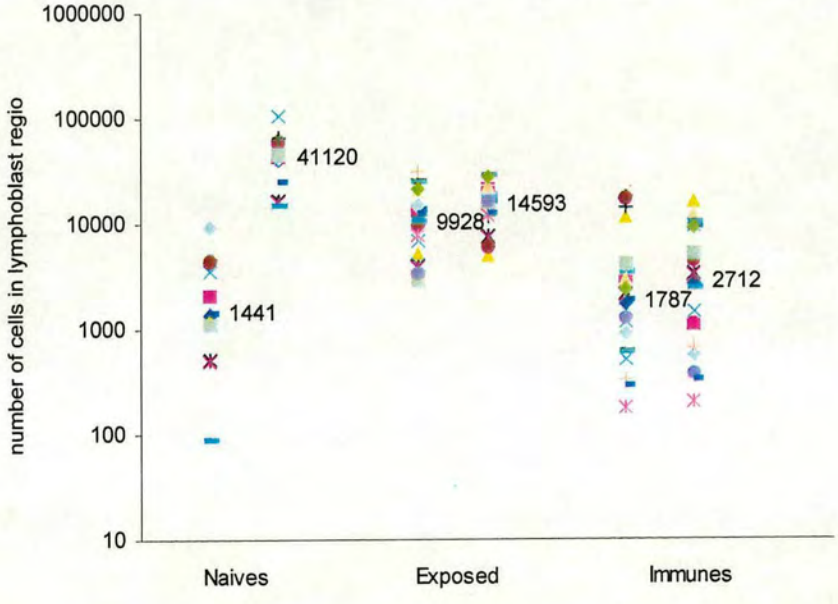


Figure 7.2: Comparison of percentages of the lymphoblast population (R2) which are a) T lymphocytes (CD3+), b) B lymphocytes (CD20+) and c) NK cells (CD56+) in uRBC-stimulated (left hand columns) and PfSE-stimulated (right-hand columns) cultures between naïve, exposed and immune individuals on **day 7**.

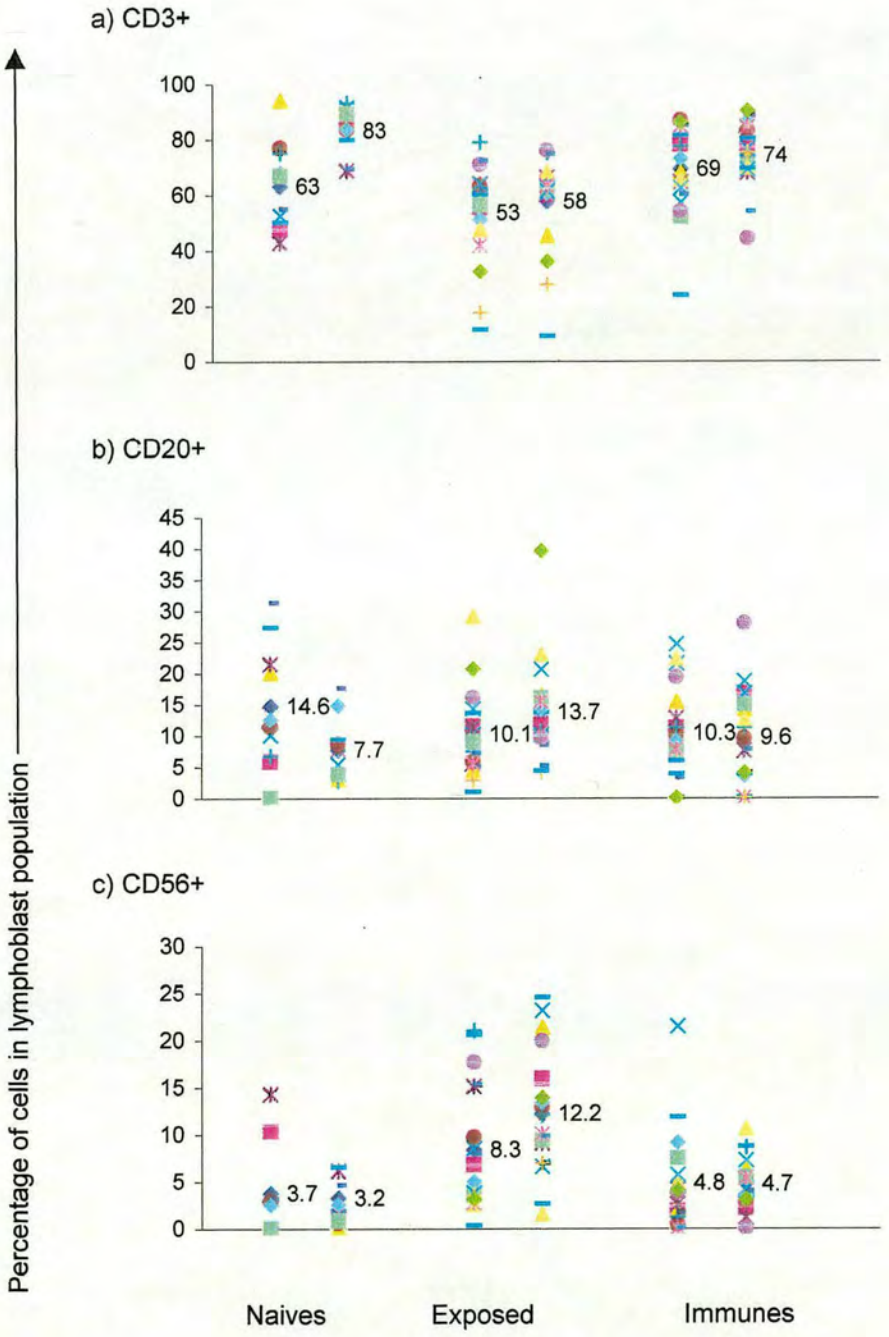


Table 7.8: Phenotype of T lymphoblasts (percentage of all lymphoblasts) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve and b) exposed donors for **day 7** (arithmetic mean and SEM indicated).

a) naives

	uRBC-stimulated		PfSE-stimulated				
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**n***	**t**	**p**
CD3+ CD45RA+	35.1	7.1	16.5	1.0	10	2.60	0.029
CD3+ CD45RO+	18.6	6.5	72.0	3.5	10	7.89	<0.001
CD3+ TcRαβ+	26.0	5.1	52.8	4.7	8	6.69	<0.001
CD3+ TcRγδ+	2.2	1.1	11.5	0.8	9	9.95	<0.001
CD3+ CD4+	31.8	4.3	79.4	2.6	10	11.43	<0.001
CD3+ CD8+	8.5	2.0	2.2	0.6	9	3.12	0.014

b) exposed

	uRBC-stimulated		PfSE-stimulated				
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**	**n***	**t**	**p**
CD3+ CD45RA+	28.2	2.7	19.5	2.4	18	5.01	<0.001
CD3+ CD45RO+	31.6	3.3	42.9	3.4	18	5.10	<0.001
CD3+ TcRγδ+	5.5	1.2	5.7	0.8	17	0.15	>0.05
CD3+ CD4+	40.3	3.5	43.6	3.5	18	1.26	>0.05
CD3+ CD8+	26.1	2.9	25.6	3.1	18	0.14	>0.05

*Some individuals did not have enough cells for the full set of staining combinations.

SEM- Standard error of the mean.

n- number of samples/individuals on given day.

t- t value for the significance level.

p- p value.

Table 7.9: Phenotype of T lymphoblasts (total numbers of all lymphoblasts) compared between uRBC-stimulated and PfSE-stimulated cultures for a) naïve and b) exposed donors for **day 7** (geometric mean and 95% CI indicated, cell count is per 10^6 cell plated out on day 0).

a) naives

	uRBC-stimulated			PfSE-stimulated					
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**n***	**t**	**p**
CD3+ CD45RA+	415	150	1148	6654	3978	11131	10	8.43	<0.001
CD3+ CD45RO+	80	9	738	29271	17234	49714	10	6.65	<0.001
CD3+ TcRαβ+	356	96	1324	24522	13063	46036	8	9.15	<0.001
CD3+ TcRγδ+	5	1	25	4898	2876	8343	9	11.10	<0.001
CD3+ CD4+	420	139	1265	32472	19793	53274	10	11.77	<0.001
CD3+ CD8+	80	13	508	773	436	1371	9	3.01	0.017

b) exposed

	uRBC-stimulated			PfSE-stimulated					
Cell Phenotype	**Mean**	**CI**		**Mean**	**CI**		**n***	**t**	**p**
CD3+ CD45RA+	2507	3502	1794	2450	3243	1851	18	0.21	>0.05
CD3+ CD45RO+	2787	3586	2167	5689	7759	4172	18	4.77	<0.001
CD3+ TcRγδ+	421	660	268	712	1080	470	17	3.01	0.008
CD3+ CD4+	3598	4831	2680	5748	7715	4282	18	2.97	0.009
CD3+ CD8+	2233	2994	1666	3285	4570	2361	18	3.27	0.004

*Some individuals did not have enough cells for the full set of staining combinations.

CI- Confidence interval.

n- Number of samples/individuals on given day.

t- t value for the significance level.

p- p value.

Figure 7.3: Comparison of CD4+ (a-b), CD8+ (c-d) and CD4+/CD8+ ratios (e-f) within the T lymphoblast population of uRBC-stimulated (left-hand columns) and of PfSE-stimulated (right-hand columns) cultures between naïve and exposed individuals on **day 7**. Panels a, c and e compare percentages of cells (arithmetic mean indicated), and panels b, d and f compare total numbers of cells (geometric mean indicated, cell count is per 10^6 cell harvested).

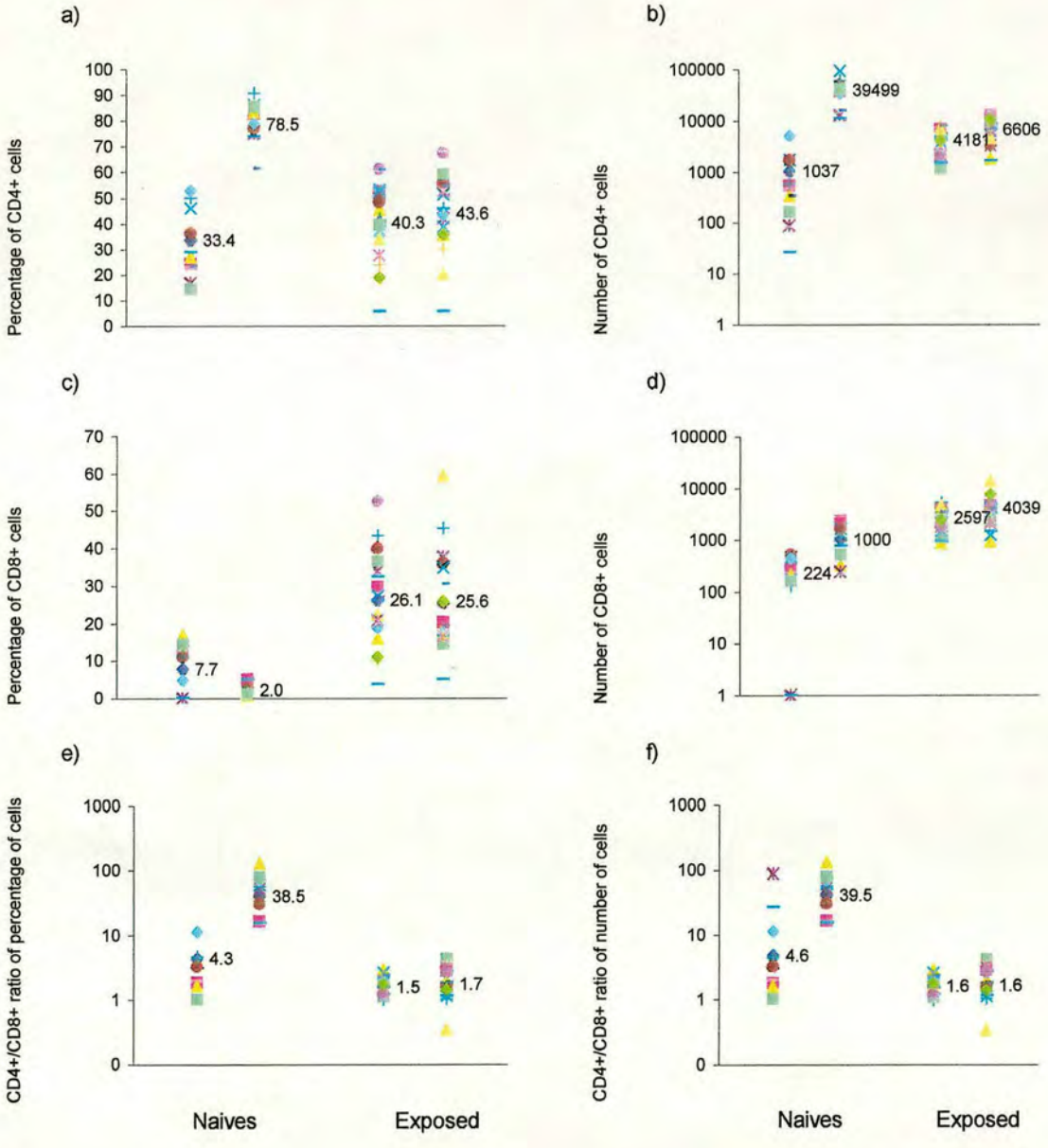


Figure 7.4: Intracellular cytokine staining to determine the cellular source of IFN-γ in PBMC from a malaria naïve donor cultured for 7 days with either SEB (panels a, d, g), PfSE (b, e, h) or uRBC (c, f, i). Lymphocytes were divided into resting cells (R1) (panels d-f) and lymphoblasts (R2) (panels g-i) on the basis of forward and side scatter (panels a to c) and stained for CD3 (vertical axis) and IFN-γ (horizontal axis).

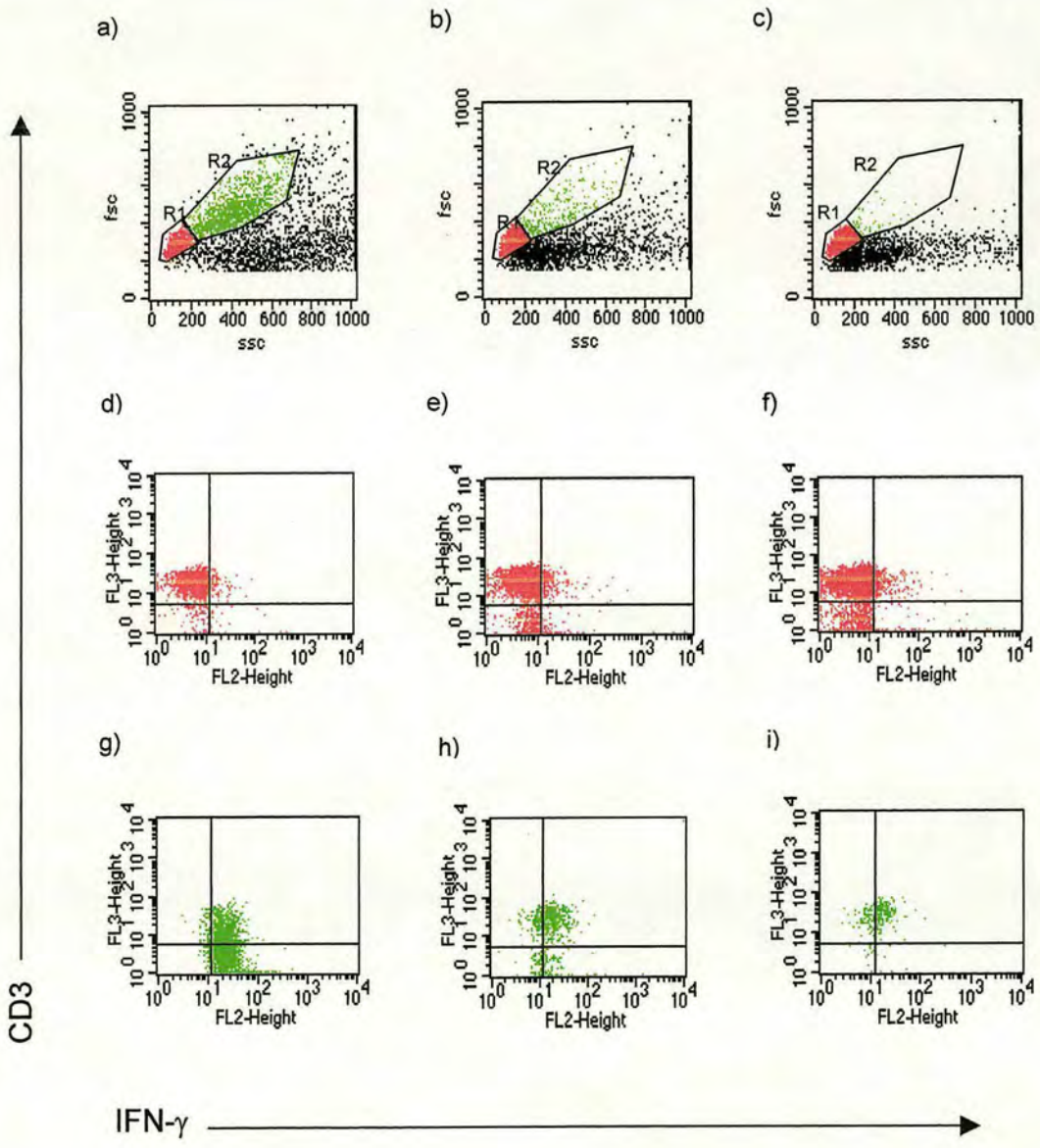


Table 7.10: Percentages (a) and absolute cells numbers (b) of total cells recovered which were lymphoblasts and the percentage of total number of lymphoblasts that were stained positive for IFN-γ comparing uRBC-stimulated and PfSE-stimulated cultures in naïve individuals on **day 7**(arithmetic mean and SEM indicated, n = 3, cell count is per 10^6 cell plated out on day 0).

a) Percentages

	uRBC-stimulated		PfSE-stimulated	
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**
R2	1.6	0.3	4.7	1.0
CD56+	5.2	3.7	18.6	5.0
CD56+/IFN-γ+	5.2	4.0	18.1	5.4
CD3+	89.3	2.5	83.0	1.7
CD3/IFN-γ+	56.0	4.0	56.6	7.4
TcR αβ+	76.8	2.6	65.5	6.9
TcR αβ+/IFN-γ+	57.6	2.3	51.3	9.2
TcR γδ+	8.6	1.9	19.6	3.4
TcR γδ+/ IFN-γ+	9.1	2.0	21.5	3.6

b) Absolute numbers

	uRBC-stimulated		PfSE-stimulated	
Cell Phenotype	**Mean**	**SEM**	**Mean**	**SEM**
R2	12640	2085	37573	8170
CD56+	816	656	7036	2608
CD56+/IFN-γ+	826	703	6792	2707
CD3+	11197	1535	31425	7208
CD3/IFN-γ+	6907	583	22439	6770
TcR αβ+	9751	1750	25127	6567
TcR αβ+/IFN-γ+	7282	1233	20330	6444
TcR γδ+	1129	372	7549	2267
TcR γδ+/ IFN-γ+	1186	377	8279	2446

SEM- Standard error of the mean.

Chapter 8: Conclusions and future directions

8.1 Summary of the study findings

The specific aims of this study were to determine whether or not there were differences in cellular proliferation, cytokine production and cell subset expansion in response to *P. falciparum* Ag *in vitro* between malaria-naïve, malaria-exposed (semi-immune) and clinically immune individuals, and if such differences were observed, to determine how these differences relate to the development of clinical immunity. These questions were answered by developing specific and sensitive methods for detecting IFN-γ, IL-12 and IL-10, by determining the cellular source of IFN-γ and its regulation and by comparing proliferative and cytokine responses between these groups.

Two-site capture ELISAs were developed to detect IFN-γ, IL-12 p70, IL-12 p40 and IL-10. Sandwich ELISAs were specific for these human cytokines but were only sensitive enough to measure pg amounts of cytokine. Because IL-12 was not easily detected by ELISA, qualitative RT-PCR was also developed to measure IL-12 p40 transcripts. Although IL-12 bioactivity was clearly demonstrated by IL-12 neutralisation assays, neither ELISA nor RT-PCR could consistently detect parasite-induced IL-12.

Individuals were allocated to naïve, exposed and immune populations before any immunological analyses were performed. Malaria-specific Ab measurements and parasite detection by blood smear or PCR confirmed the appropriate designation of individuals into each group (see table 2.2). Immunological responses were then measured by PfSE-stimulation of PBMCs *in vitro*. Peripheral blood was chosen as the source of lymphocytes because of practical constraints and also because peripheral blood is believed to represent the total malaria-reactive lymphocyte population in healthy individuals.

Cells from all individuals responded vigorously to PfSE by lymphocyte proliferation, but there were no significant differences in geometric mean SI between any of the groups. Although malaria-specific Ab levels were high in immune individuals, Ab levels were only correlated with proliferative responses in exposed individuals. Further, IFN-γ levels were correlated with proliferative responses in each group,

although when all individuals were grouped together, there was no correlation between these parameters.

PBMCs from naïve individuals produced moderate levels of IFN-γ in response to PfSE stimulation and production was, for the most part, IL-12-dependent. Additionally, αβ+ T cells were the predominant cells which produced IFN-γ, although significant proportions of γδ+ T cells and NK cells also produced IFN-γ. Cells from exposed individuals produced significantly higher mean IFN-γ levels than PBMCs from naïve individuals, and IFN-γ levels were only partially reduced by neutralising αIL-12. In contrast, cells from immune individuals secreted marginal levels of IFN-γ which were significantly lower than levels found in either naïve or exposed populations, and neutralising αIL-12 reduced IFN-γ to background levels. Low IFN-γ responses in immune individuals were not due to anergy, as their cells proliferated in response to PfSE to the same extent as cells from naïve and exposed individuals. IL-10 was measured in order to determine if this cytokine was involved in the downregulation of the IFN-γ response. Although the number of IL-10 producers was highest in exposed individuals, there were no significant differences in malaria-specific IL-10 levels between any of the study populations.

Finally, in all individuals, cells which responded to PfSE were predominantly T cells, although there were significant proportions of activated NK cells in cell cultures of naïve and exposed individuals and higher proportions of activated B lymphocytes in cell cultures of exposed and immune populations than naïve individuals. Naïve individuals had significantly higher numbers of PfSE-stimulated cells than exposed and immune individuals. Parasite-stimulated cells from naïve individuals were predominantly CD4+ αβ+ CD45RO+ cells, although γδ+ and CD45RA+ T cells also responded to PfSE. In contrast, in exposed individuals, significantly higher numbers of CD8+ cells were activated than in naïve individuals.

8.2 Practical constraints of measuring cellular immunity in humans

Assays, such as measuring malaria-specific Ab levels, are able to directly monitor immune responses during an acute infection with malaria. Because invasive techniques are not practical or ethical in humans, mouse models are often used to determine immune responses in localised areas (i.e. liver or spleen). However, murine

models are, at best, only models and cannot be completely conclusive on how the human immune system responds to malaria infection. *In vitro* assays are also limited by the fact that simply removing and manipulating cells outside their natural environment may cause changes in how cells react, thus making comparisons between different studies difficult (282). Despite the limitations of *in vitro* assays, measuring peripheral blood responses was the only way in which to compare cellular immune responses in healthy individuals in this study. Although not all assays were performed at the same time with the same batch of malaria Ag, all of these differences were accounted for and there was little variation between assays. Thus, some initial conclusions can be made from these *in vitro* assays as to what happens during malaria infection *in vivo*.

8.3 Clinical immunity: hypothesis revisited

Severe clinical symptoms of malaria are associated with elevated levels of pro-inflammatory cytokines such as TNF-α (118, 154, 193). It was originally thought that parasite-derived endotoxins directly stimulate macrophages to produce TNF-α. However, the idea that this innate immune response is sufficient to mediate pathology cannot explain many of the epidemiological characteristics of malaria infections, including why young infants do not become ill when first infected with malaria (279). I have hypothesised that IFN-γ is needed to stimulate excessive levels of pro-inflammatory cytokines which cause pathology and that the acquisition of clinical immunity is dependent upon the downregulation of this inflammatory response. Although the innate immune response, mediated by Mø and NK cell production of pro-inflammatory cytokines, may be important during malaria infection, it is likely that the inflammatory response is dependant upon an early T cell response primed by malaria Ag or other cross-reacting environmental antigens. To date, these hypotheses are confirmed by 3 studies which show that IFN-γ levels are lower in malaria-immune adults than in unexposed individuals (53, 284) and that malaria Ag-stimulated TNF-α production is dependent upon CD3+ cells (308).

The results from this study further confirm these hypotheses. Cells from naïve individuals proliferated vigorously in response to malaria Ag and secreted significant amounts of IFN-γ. Upon PfSE stimulation, responding cells from naïve individuals

were predominantly CD3+ CD4+ αβ+ CD45RO+ but NK, αβ+ and γδ+ cells all produced IFN-γ. It was also found that naïve individuals had a significantly higher number of PfSE-stimulated lymphoblasts than exposed and immune individuals. Taken together, these results suggest that a polyclonal expansion of primed αβ+ and γδ+ T cells stimulate excessive pro-inflammatory responses leading to a febrile illness in naïve individuals. Together with αβ+ and γδ+ cells, NK cells also produce IFN-γ which may stimulate macrophages to produce TNF-α, IL-1 and IL-6. In naïve individuals, there was no evidence of production of anti-inflammatory cytokines (such as IL-10) or of a Th2-like immune response which may ameliorate the pro-inflammatory response (196). It has been suggested that naïve individuals have primed CD4+ CD45RO+ cells, which are activated by cross-reactive memory responses to other environmental organisms (62, 63). If so, this would explain why foreign adults and young children living in endemic areas are more susceptible to severe malaria than young infants whose immune systems have never been previously primed to malaria.

Exposed individuals, who are still susceptible to clinical malaria (but at lower risk of severe disease compared with naïve individuals), have significantly higher levels of IFN-γ in response to malaria Ag. Although the cellular source of IFN-γ was not determined in exposed individuals, it could be that CD8+ cells are the primary producers of IFN-γ, as this population was markedly expanded in exposed individuals. CD8+ cells in exposed individuals may be generating a malaria Ag-specific response while the polyclonal activation of CD4+ cells to numerous cross-reactive antigenic epitopes may be downregulated. Thus, the severity of infection does not only depend on inflammatory cytokine concentrations, but may also depend upon which cells are producing these cytokines and where they are being produced. Although IFN-γ is beneficial in clearing primary malaria infections (93, 98), excessive levels can cause severe pathology (181, 350). Because exposed individuals are not necessarily at higher risk of severe malaria than naïve individuals, there may be mechanisms which ameliorate the downstream effects of higher levels of IFN-γ. Although PfSE-stimulated PBMCs of exposed individuals did not produce significant levels of IL-10, exposed individuals had a much higher proportion of B cells than naïve individuals and a relatively high concentration of malaria-specific antibodies. This may be indicative of a

switch in the immune response away from a strong Th1 profile towards a Th1/Th2 or Th2-dominated response.

Finally, in clinically immune individuals, there is downregulation of inflammatory cytokines as evidenced by the fact that cells from immune individuals produced very low levels of IFN-γ. Because ICS analysis was not performed for cells from immune individuals, the cellular source of IFN-γ is not known. However, it could be hypothesised that NK cells are the primary producers of IFN-γ in immune individuals, because IFN-γ was completely IL-12-dependent (144, 301) in immune individuals but not in naïve or exposed individuals. Low numbers of NK cells in the responding cell population in conjunction with marginal production of IFN-γ in immune individuals give further support to this hypothesis. Thus, a plausible response to malaria infection in immune individuals is: (i) that there may be an immediate production of IFN-γ by NK cells which may be sufficient to induce IFN-γ-dependent parasite killing, but that (ii) this initial innate immune response is quickly downregulated and production of Mø-derived TNF-α is inhibited by malaria-specific T cells which produce anti-inflammatory cytokines and provide help to B cells for malaria-specific Ab production. Again, there were no significant differences in IL-10 levels between any of the groups, suggesting that other anti-inflammatory cytokines, such as TGF-β, may be involved.

In conclusion, the acquisition of clinical immunity to malaria seems to be due to a downregulation of the inflammatory response which is mediated both by the innate immune system and by previously primed T cells. However, clinical immunity is not completely a 'Th1-like' or 'Th-2' immune response. As in other parasitic infections, a combination of inflammatory and anti-inflammatory cytokines are needed to help reduce parasitemia and ultimately to develop clinical immunity (173, 298). Rather it is the timing, location, cellular source of cytokine production, as well as the balance in cytokine levels, which determine the outcome of malaria infection.

The practical implications to consider from these results are how new therapies can be developed and what factors are important in vaccine development (see section 8.3.1). Therapies which help to downregulate an excessive inflammatory response, and in a sense 'mimic' the immune response found in clinically immune individuals, would be beneficial in developing anti-toxic immunity. It is also important to understand when a therapy such as this can be used as results from this study seem to suggest

that an early moderate inflammatory response may be beneficial (i.e. IFN-γ production from NK cells seems to persist in all 3 groups). However, this study has only begun to elucidate some of the mechanisms involved in the immune response to malaria infection. There are many questions which still remain to be answered. The following section describes some of the main ideas which need to be further investigated in order to gain a more thorough understanding of clinical immunity to malaria.

8.4 Future studies

8.4.1 Which component(s) of the malaria parasite activates the immune system?

The exact parasite component or components which stimulate cells are still not known. In one study, the T cell activating component of malaria parasites was found to be distinct from the TNF-α inducing endotoxin-like Ag (75) used in other studies (305, 328). Studies performed previously in our laboratory also demonstrated that live schizonts stimulate a higher percentage of γδ+ cells to respond than does a crude Ag preparation of schizont extracts (354). Taken together, these results suggest that there are multiple components of the malaria parasite which stimulate different immune responses.

More importantly, it is not known whether differences in immune responses between naïve and immune individuals are a result of cells responding to different antigenic components. Cells from malaria-exposed individuals proliferated vigorously in response to separated protein fractions of *P. falciparum* schizonts (114) and purified soluble malaria Ag (284) while cells from malaria-naïve individuals did not (114, 284). These results suggest that if soluble crude malaria Ag is affinity purified with antibodies from immune individuals, cross-reactive antigens are lost and malaria specific responses are maintained. CD4+ cells seem to respond to Ag expressed by schizonts and by many other environmental organisms. But data in this study from malaria-exposed individuals seem to suggest that CD8+ cells may respond more specifically to malaria-specific Ag. These results have important implications in vaccine development, because different malaria antigens will stimulate different cellular subsets leading to production of cytokines which will either prevent or exacerbate severe malaria. The components which stimulate cells from immune individuals may be used to generate Ag-specific responses in a malaria vaccine.

8.4.2 Why was IL-12 not detected?

8.4.2.1 IL-12 receptor expression

There were no significant differences found in IL-12 levels in cell cultures of naïve, exposed and immune populations, and no obvious differences were found in IL-12 mRNA transcription. However, IL-12 activity was demonstrated by assays in which IFN-γ production was down-regulated in the presence of antibodies which neutralised IL-12. The question that remains to be answered is; why were differences in IL-12 levels not detected? It could be that IL-12 production is very transient, and the timepoints when samples were collected were not representative of when IL-12 was actually being produced. Another reason may be that IL-12 is produced and is biologically active in such miniscule amounts that the assays were not sensitive enough to detect mRNA or protein levels.

However, it could also be that the IL-12 levels measured were the true levels produced, but that there are other factors, such as IL-12R expression, which regulate IL-12 bioactivity. Ouyang *et al.* demonstrated that IL-12R β2 subunit expression is downregulated by IL-4 (323) and GATA-3, a transcription factor which augments IL-4 and IL-5 production (254). Although PBMCs from all 3 groups generally produced equal amounts of IL-12, the cytokine milieu in immune individuals (i.e. Th2 cytokines) may help to downregulate IL-12R expression on NK or T cells. Reduced IL-12R expression could also explain why cells from immune individuals produce low levels of IFN-γ, as most of their IFN-γ production is dependent upon IL-12. Thus, even if there is IL-12 produced, absolute concentrations may not be important if other factors mediate IL-12R expression.

8.4.2.2 Dendritic cells

Again, there may have been differences in IL-12 production, but these differences may not have been detected because the wrong population of cells were examined. One possible population of cells that was not specifically examined is dendritic cells (DC). Because DC are such a small percentage of peripheral blood, the IL-12 signal may have been reduced by the presence of other cells which do not produce IL-12 (i.e. the assays were not sensitive enough). Purified mouse and human

DC are potent producers of IL-12 (50, 177) and were found to produce comparable amounts of IL-12 as PBMCs, spleen cells and macrophages on a cell to cell basis (133). IL-12 was primarily produced by DC, not Mø, in murine *T. gondii* infection (276). It was further demonstrated that DC were more effective transporters of Ag to T cell areas than Mø [Reis e Souza, 1997 #446], that they mediate Th1 differentiation and production of IFN-γ from Th1 cells (133) and that anti-inflammatory cytokines downregulate IL-12 production by DC (177). These results could explain why T cells from naïve individuals produce IFN-γ early on while there is no such response found in immune individuals. Although no such studies have been carried out in malaria infections, these results suggest that DC may be more important producers of IL-12 than Mø during malaria infection. Further studies using purified PfSE-stimulated DC and Mø are needed to determine if this is the case.

8.4.3 Factors which mediate cytokine production other than IL-12

Although IL-12 does play an important role in malaria infection as evidenced by the neutralisation assays with αIL-12, there may be other factors which are important in determining whether the inflammatory response is excessive or downregulated.

8.4.3.1 Co-stimulatory molecules

Although MHC-T cell interaction stimulates cells to produce cytokines, it has been demonstrated that the additional binding of co-stimulatory ligands increases the production of cytokines such as IL-12 (50, 177), IFN-γ (143) and TGF-β (52). CD40/CD40 ligand (CD40L) binding combined with IL-12 (259), and B7 interacting with CD28 receptors on NK cells (143), increase IFN-γ concentrations. Although no work has been done in human malaria models on co-stimulatory molecules, these interactions could cause the increase in IFN-γ levels found in cells from naïve and exposed individuals and not from immune individuals.

Alternatively, it could also be that other co-stimulatory molecules are preferentially stimulated which increase concentrations of anti-inflammatory cytokines or downregulate inflammatory cytokines in immune individuals. Engagement of B7-2 stimulates the production of IL-4 (104), and engagement of CTLA-4/CD28 induces TGF-β and has been found to decrease proliferative responses and IFN-γ production by

T cells (52). CD40/CD40L stimulates both Th1 and Th2 cytokines (259, 271), but is absolutely needed for optimal production of Th2 cytokines (271).

Additionally, co-stimulatory molecules are bi-directional in that they stimulate antigen presenting cells as well as T cells. Ligation of CD40 stimulates growth and differentiation of B cells, isotype switching and stimulates Mø production of IL-1 and TNF-α (259). Thus an Ab-mediated or pro-inflammatory mediated response may depend on the type of antigen presenting cell. Further studies are required to determine if there are differences in co-stimulatory molecules and antigen presenting cells which present malaria antigens in naïve, exposed and immune populations.

8.4.3.2 Other inflammatory cytokines: IL-15 and IL-18

In this study, it has been demonstrated that IL-12 regulates IFN-γ production. However, neutralising αIL-12 only partially decreased IFN-γ levels in cells from exposed individuals and some naïve individuals, suggesting either that there are IL-12 independent cellular sources of IFN-γ or that other cytokines also mediate IFN-γ production. Both IL-15 and IL-18 are produced by Mø and may play an important role in stimulating the inflammatory response during malaria infection. IL-15 is a potent stimulator of IFN-γ from NK cells (160, 325) and γδ+ T cells (106). IL-15 is more important than IL-2 in localised stimulation of T cells (325) and has also been shown to increase the cytolytic function of CD8+ cells (160). It could be that the increase in the absolute numbers and proportions of CD8+ cells found in exposed individuals is mediated by IL-15 production and that the expansion of these CD8+ cells is indicative of a beneficial Ag-specific IFN-γ-mediated response in localised areas where infection is present.

IL-18 also stimulates IFN-γ production from NK cells (179, 232, 249, 250). It is a more potent inducer of IFN-γ than IL-12 and may be the cytokine which is stimulating IL-12 independent IFN-γ from cells of some naïve and exposed individuals (249). IL-18 can also synergise with IL-12 to enhance IFN-γ levels (232). Although no studies have measured IL-15 and IL-18 levels in malaria models, it is possible that the insignificant differences detected in IL-12 production between cells of naïve, exposed and immune populations are real; levels of IL-12 produced are similar in all groups and that

differences in IL-15 or IL-18 may be more important than IL-12 in regulating IFN-γ production by cells from naïve and exposed individuals.

8.4.4 How is the inflammatory response downregulated?

This project primarily focused on the role of inflammatory cytokines and only hypothesised on what role anti-inflammatory cytokines may play in malaria infection. This study has led to the suggestion that one of the mechanisms which is involved in the development of clinical immunity to malaria is the downregulation of inflammatory cytokines. IL-10 levels were low or undetectable in all cultures and no differences were observed between groups, suggesting that IL-10 does not play a major role in the downregulation of IFN-γ. A prime candidate which can downregulate inflammatory responses that has not been examined in these individuals is TGF-β. TGF-β levels are inversely correlated with the degree of severity in murine malaria (252) and lower circulating levels of TGF-β have been found in individuals with acute *P. falciparum* malaria than in convalescent patients (355). TGF-β can either directly downregulate IL-12 mediated IFN-γ production by reducing IL-12R expression on T cells [Gorham, 1998 #447] or downregulate the downstream effects of IFN-γ without necessarily affecting IFN-γ concentrations. In mice, TGF-β was found to decrease TNF-α concentrations without having any significant effect on IFN-γ levels (252). If TGF-β is upregulated in exposed individuals this may explain why exposed individuals can produce significantly higher levels of IFN-γ without necessarily being more susceptible to severe malaria than naïve individuals.

TGF-β is pro-inflammatory at low concentrations and anti-inflammatory at high concentrations (251). Thus, a balance in concentration, location and timing of TGF-β production and activation will determine whether TGF-β is pathogenic or not. If low levels of TGF-β can be stimulated early during acute *P. falciparum* infection, it could possibly aid parasite clearance by stimulating pro-inflammatory cytokines and upregulating FcγRIII on monocytes leading to the phagocytosis of infected RBCs (251). Later on in infection, high doses of TGF-β could down-regulate excessive inflammatory cytokines and thus prevent much of the immunopathology.

8.4.5 The cellular response in the exposed and immune individuals

A more thorough examination needs to be carried out in immune individuals of which cellular subsets are expanded in response to PfSE. Although the proportions of T, B and NK cells were examined in cells from immune individuals, it is not known whether they also have a similar expansion of CD8+ cells as found in exposed individuals. If immune individuals do make a CD8+ response, it would imply that Ag-specific T cell IFN-γ production is beneficial and that IFN-γ production from polyclonally activated CD4+ cells (such as in naïve individuals) may be harmful. On the contrary, if immune individuals did not have a similar expansion of CD8+ cells, and as innate NK cell IFN-γ responses seems to persist in all 3 groups, it may be that IFN-γ production by T cells may exacerbate infection whereas IFN-γ production by NK cells is beneficial.

Although it was determined that NK, αβ + and γδ+ cells produce IFN-γ in naïve individuals, it is still unknown which cell populations produce IFN-γ in exposed and immune individuals. In order to answer these questions, it is important to take time in choosing and developing the appropriate assay. Intracellular cytokine staining is a method which detects cytokine production at the single cell level. Although ICS cannot measure the exact concentrations of cytokines produced, it can determine the numbers and phenotype of cytokine producing cells. This approach may in fact be better than ELISA assays which can only measure the sum total of production, consumption and degradation of secreted cytokine, and thus may not represent the total amount of biologically active cytokine produced (47, 99, 357). This is a well recognised problem for measurement of cytokines which are quickly bound and internalised by cells such as IL-2 (1). Although RT-PCR is also used to measure cytokine concentrations, RNA transcripts do not necessarily correlate with the amount of protein synthesised (47, 99, 357). A good example of this is TGF-β where most cells constitutively express TGF-β transcripts, and its activity is modulated by post-translational processing of an inactive precursor molecule (348). However, ICS assays need to be carefully optimised for each cytokine measured. It has been found that different reagents, concentrations of reagents and incubation times (83, 246) need to be used depending on the cytokine measured as well as the type of cell being examined (83, 303).

8.4.6 Genetic regulation

Although immune individuals generally had low IFN-γ levels, cells from a number of immune individuals produced significant levels of IFN-γ. Because there were no apparent sociological differences between any of the individuals, it could be that differing levels of IFN-γ reflect different levels of immunity. Alternatively, it could be hypothesised that these individuals are all equally immune but that there are different strategies for attaining clinical immunity (i.e. decreasing IFN-γ itself or its downstream effects). A last consideration is that there may also be genetic differences which control the immune response.

A number of studies support the idea that differences in genetic regulation control immune responses. Differences were found in the susceptibility to *P. yoelii* between different strains of mice (310). In humans, many polymorphisms have been described in cytokine genes which have been shown to affect cytokine levels and disease susceptibility. For example, high IL-10 production was correlated with certain haplotypes (90). Additionally, children who were homozygous for TNF2, an allele of the TNF-α gene promoter region, were more susceptible to cerebral malaria (224). Humans are not genetically identical, thus more work needs to be done in order to determine if there are heritable factors which cause each individual to respond differently to malaria infection.

In conclusion, malaria is a complex disease which stimulates different immunological responses depending on levels of acquired immunity. There is likely to be a critical balance in the site and the timing of cytokine production and in the cellular sources of cytokines which determines if the immune response is beneficial or pathogenic. Although I have begun to elucidate some of the immune mechanisms involved in *P. falciparum* infection, not enough is known about how immune mediators function in malaria infections. If serious thought is to be given to malaria vaccine development, more studies need to be done on the critical immune mechanisms which mediate both protection and clinical immunity.

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Appendix 1

Table A1.1: Complete RT-PCR results for naïve study population.
no RT= no reverse transcription.
+ = band of correct size detected.
- = no band/ no band of correct size.
ND= not done.

a) N1

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	+	+	+	-
RBC	IL-12	-	+	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	+	+	-	-
PfSE	IL-12	+	+	+	+	-
PfSE-no RT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	ND
	IL-12	ND
Plasmid	β-actin	ND
	IL-12	ND

b) N2

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	+	+	-	+	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	+	+	+	-	+
PfSE-no RT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	ND
	IL-12	ND
Plasmid	β-actin	ND
	IL-12	ND

c) N3

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	-	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
PHA	β-actin	+	+	+	+	+
PHA-no RT	β-actin	-	-	-	+	+
PHA	IL-12	+	-	-	-	-
PHA-no RT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

d) N4

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	+	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	+	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
PHA	β-actin	+	+	+	+	+
PHA-no RT	β-actin	-	-	-	-	-
PHA	IL-12	+	-	-	-	-
PHA-no RT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

e) N5

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	+	-
PHA	β-actin	+	+	+	+	+
PHA-no RT	β-actin	-	-	-	-	-
PHA	IL-12	-	-	-	-	-
PHA-no RT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

f) N12

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	+	-	-	-
RBC-no RT	IL-12	+	-	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	-	+	-
PfSE-no RT	IL-12	-	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	+	-	+	-
LPS	IL-12	-	-	-	+
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

g) N13

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	+	-
RBC	IL-12	-	-	+	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	-	+	-
PfSE-no RT	IL-12	-	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	-	+	+	+
LPS-noRT	IL-12	+	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

h) N14

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	-	-	-	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	-	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	+	-	-
PfSE-no RT	IL-12	-	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	+	+	-	-
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Table A1.1 (continued):

i) N15

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	-	-	-	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	-	+	-
PfSE-no RT	IL-12	-	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	+	-	-	-
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

j) N16

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	+	-	+	-
RBC-no RT	IL-12	+	+	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	-	-	-
PfSE-no RT	IL-12	+	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	+	-	+	+
LPS-noRT	IL-12	-	-	-	+

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

k) N17

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	+	+	-	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	+	+	-	-
PfSE-no RT	IL-12	-	-	+	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	+	-	-	-
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

l) N18

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	-
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	-	-	-	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	-	-	+	-
PfSE-no RT	IL-12	+	-	+	+
LPS	β-actin	ND	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	-	+	-	-
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

m) N19

Stimulus	cDNA	Day 1	2	4	6
RBC	β-actin	+	+	+	+
RBC-no RT	β-actin	-	-	-	-
RBC	IL-12	-	-	-	-
RBC-no RT	IL-12	-	-	-	-
PfSE	β-actin	-	+	+	+
PfSE-no RT	β-actin	-	-	-	-
PfSE	IL-12	+	-	-	-
PfSE-no RT	IL-12	-	-	-	-
LPS	β-actin	+	+	+	+
LPS-noRT	β-actin	-	-	-	-
LPS	IL-12	+	-	-	-
LPS-noRT	IL-12	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Table A1.2: Complete RT-PCR results for exposed study population.
no RT= no reverse transcription.
+ = band of correct size detected.
- = no band/ no band of correct size.
ND= not done.

a) E1

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	-	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

b) E2

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

c) E3

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	-	-	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

d) E5

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	ND	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	+	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

e) E6

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	-

f) E7

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	ND	+
RBC-no RT	β-actin	-	-	-	ND	-
RBC	IL-12	-	-	-	ND	-
RBC-no RT	IL-12	-	-	-	ND	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

g) E8

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	-	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	+	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	+	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

h) E9

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	+	+	+	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	-	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	+	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	-	-	-	-
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	+	+	+	+	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Table A1.2 (continued):

i) E10

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	-	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

j) E11

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	+	-	+	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	-	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	+	-	+
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

k) E12

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	+	+	+	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	+	-	+
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	+	+	+	+	+
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	-

l) E14

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	-
	IL-12	+

m) E15

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	+	-	-	+
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	+	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	+
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

n) E16

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	ND	+
LPS-noRT	β-actin	-	-	-	ND	-
LPS	IL-12	-	-	-	ND	-
LPS-noRT	IL-12	-	-	-	ND	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

o) E17

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	-	-	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	-
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	-
	IL-12	+

p) E18

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	+	-	+
RBC	IL-12	+	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	+	-	-	-	-
PfSE	IL-12	+	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	+	+	+	-
LPS	IL-12	+	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Table A1.2 (continued):

q) E19

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

r) E20

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	-	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

s) E21

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	+	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin		-	-	-	-
LPS	IL-12	+	-	+	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

t) E22

Stimulus	cDNA	Day 1	2	4	6	8
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	+	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	+	+	+	+	+
LPS-noRT	β-actin	-	-	-	-	-
LPS	IL-12	+	-	-	-	-
LPS-noRT	IL-12	-	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Table A1.3: Complete RT-PCR results for control individuals.
no RT= no reverse transcription.
+ = band of correct size detected.
- = no band/ no band of correct size.
ND= not done.

a) C2

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	ND	+	+	+	+
LPS-noRT	β-actin	ND	-	-	-	-
LPS	IL-12	ND	-	-	-	-
LPS-noRT	IL-12	ND	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

b) C3

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	ND	+	+	+	+
RBC-no RT	β-actin	ND	-	-	-	+
RBC	IL-12	ND	-	-	-	-
RBC-no RT	IL-12	ND	-	-	-	-
PfSE	β-actin	ND	+	+	+	+
PfSE-no RT	β-actin	ND	-	-	-	-
PfSE	IL-12	ND	-	-	-	-
PfSE-no RT	IL-12	ND	-	-	-	-
LPS	β-actin	ND	+	+	+	+
LPS-noRT	β-actin	ND	+	-	-	-
LPS	IL-12	ND	-	-	-	-
LPS-noRT	IL-12	ND	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

c) C4

		Day				
Stimulus	**cDNA**	**1**	**2**	**4**	**6**	**8**
RBC	β-actin	+	+	+	+	+
RBC-no RT	β-actin	-	-	-	-	-
RBC	IL-12	-	-	-	-	-
RBC-no RT	IL-12	-	-	-	-	-
PfSE	β-actin	+	+	+	+	+
PfSE-no RT	β-actin	-	-	-	-	-
PfSE	IL-12	-	-	-	-	-
PfSE-no RT	IL-12	-	-	-	-	-
LPS	β-actin	ND	+	+	+	-
LPS-noRT	β-actin	ND	-	-	-	-
LPS	IL-12	ND	-	-	-	-
LPS-noRT	IL-12	ND	-	-	-	-

Controls		
Blank	β-actin	-
	IL-12	-
Blank-cDNA	β-actin	-
	IL-12	-
Plasmid	β-actin	+
	IL-12	+

Appendix 2

Table A2.1: Complete lymphoproliferative results for PBMCs of naïve individuals by day. Data is given in geometric mean cpm (left-hand tables), and SI (PfSE = PfSE/uRBC, PHA = PHA/uRBC, right-hand tables)(ND= not done).

a) Day 1

Donor	RBC	PfSE	PHA
N1	398	331	547
N2	303	174	470
N3	667	600	794
N4	908	830	1055
N5	600	489	1606

Donor	PfSE	PHA
N1	1	1
N2	1	2
N3	1	1
N4	1	1
N5	1	3

b) Day 2

Donor	RBC	PfSE	PHA
N1	431	794	72158
N2	221	491	71656
N3	730	1048	38149
N4	1102	1101	59626
N5	359	466	32188

Donor	PfSE	PHA
N1	2	167
N2	2	324
N3	1	52
N4	1	54
N5	1	90

c) Day 3

Donor	RBC	PfSE	PHA
N1	89	2282	41677
N2	86	2519	31224
N3	1093	1606	72535
N4	925	1274	92917
N5	479	1934	91055
N6	794	911	40866
N7	576	642	55953
N8	488	1252	38725
N9	515	853	41170
N10	498	717	57674
N11	561	740	47383
N12	406	805	58313
N13	246	375	16725
N14	144	187	24496
N15	233	318	18379
N16	47	111	14449
N17	39	50	19073
N18	85	394	19200
N19	22	84	27230

Donor	PfSE	PHA
N1	26	467
N2	29	365
N3	1	66
N4	1	100
N5	4	190
N6	1	51
N7	1	97
N8	3	79
N9	2	80
N10	1	116
N11	1	85
N12	2	144
N13	2	68
N14	1	170
N15	1	79
N16	2	310
N17	1	485
N18	5	226
N19	4	1221

d) Day 4

Donor	RBC	PfSE	PHA
N1	414	5592	93653
N2	164	7249	104946
N3	1226	3083	96277
N4	930	2388	96082
N5	648	3061	72597

Donor	PfSE	PHA
N1	14	226
N2	44	639
N3	3	79
N4	3	103
N5	5	112

Table A2.1 (continued):

e) Day 5

Donor	RBC	PfSE	PHA
N1	554	11151	ND
N2	372	21461	90289
N3	1347	3881	107932
N4	731	2170	58922
N5	712	4508	53713
N6	988	2473	26546
N7	738	3071	18139
N8	1005	4388	34026
N9	685	4516	33719
N10	745	2758	21438
N11	668	4052	41493
N12	616	11322	17051
N13	543	1346	10303
N14	108	324	33284
N15	48	477	10410
N16	62	317	4427
N17	28	361	10603
N18	36	2036	7414
N19	50	940	6505

Donor	PfSE	PHA
N1	20	ND
N2	58	242
N3	3	80
N4	3	81
N5	6	75
N6	3	27
N7	4	25
N8	4	34
N9	7	49
N10	4	29
N11	6	62
N12	18	28
N13	2	19
N14	3	308
N15	10	219
N16	5	72
N17	13	379
N18	56	203
N19	19	131

f) Day 6

Donor	RBC	PfSE	PHA
N1	2183	27706	30434
N2	632	58520	49461
N3	1296	4980	47210
N4	1086	1627	16739
N5	1119	14313	27989

Donor	PfSE	PHA
N1	13	14
N2	93	78
N3	4	36
N4	1	15
N5	13	25

g) Day 7

Donor	RBC	PfSE	PHA
N1	2077	36539	11494
N2	797	49436	18652
N3	3081	14536	21516
N4	1279	13961	11928
N5	1489	22477	13941
N6	1888	10924	6813
N7	941	7339	3363
N8	1316	11270	7821
N9	1117	12160	7818
N10	1415	14039	6258
N11	1012	23123	11285
N12	749	8925	1338
N13	186	1897	1927
N14	254	4846	14967
N15	141	4513	6542
N16	142	11348	1984
N17	244	6863	10794
N18	146	4566	2708
N19	437	12457	2900

Donor	PfSE	PHA
N1	18	6
N2	62	23
N3	5	7
N4	11	9
N5	15	9
N6	6	4
N7	8	4
N8	9	6
N9	11	7
N10	10	4
N11	23	11
N12	12	2
N13	10	10
N14	19	59
N15	32	46
N16	80	14
N17	28	44
N18	31	19
N19	28	7

Table A2.1 (continued):

h) Day 9

Donor	RBC	PfSE	PHA
N1	1610	17154	1916
N2	536	26635	3439
N3	3077	13394	10386
N4	1678	15273	7033
N5	1864	10862	4162
N6	5567	9197	11343
N7	1563	45663	5012
N8	2308	55774	8834
N9	1889	18343	4114
N10	5309	23834	7007
N11	3165	44769	10712
N12	406	159	166
N13	474	566	458
N14	322	3989	4368
N15	475	4420	3490
N16	35	3619	683
N17	125	5779	3571
N18	574	3027	1672
N19	421	7212	1835

Donor	PfSE	PHA
N1	11	1
N2	50	6
N3	4	3
N4	9	4
N5	6	2
N6	2	2
N7	29	3
N8	24	4
N9	10	2
N10	4	1
N11	14	3
N12	0	0
N13	1	1
N14	12	14
N15	9	7
N16	105	20
N17	46	29
N18	5	3
N19	17	4

Table A2.2: Complete lymphoproliferative results for PBMCs of exposed individuals by day. Data is given in geometric mean cpm (left-hand tables), and SI (PfSE = PfSE/uRBC, PHA = PHA/uRBC, right-hand tables)(ND= not done).

a) Day 2

Donor	RBC	PfSE	PHA
E1	19	25	ND
E2	19	27	2017
E3	15	32	ND
E5	38	57	1146
E6	28	26	578
E7	29	66	4204
E8	15	45	2908
E9	81	164	2318
E10	48	86	3104
E11	99	115	4225
E12	24	60	2888
E14	61	76	6062
E15	26	64	4636
E16	39	58	4620
E17	23	35	1915
E18	29	68	3888
E19	28	65	1714
E20	41	74	4854
E21	21	21	841
E22	22	43	2258

Donor	PfSE	PHA
E1	1	ND
E2	1	105
E3	2	ND
E5	2	30
E6	1	21
E7	2	145
E8	3	198
E9	2	29
E10	2	64
E11	1	43
E12	3	121
E14	1	100
E15	2	179
E16	1	118
E17	2	83
E18	2	133
E19	2	61
E20	2	119
E21	1	41
E22	2	102

Table A2.2 (continued):

b) Day 3

Donor	RBC	PfSE	PHA
E1	24	42	ND
E2	21	30	8982
E3	28	118	ND
E5	42	150	6291
E6	36	83	5115
E7	67	337	9750
E8	53	134	6424
E9	80	789	4303
E10	147	646	12508
E11	77	345	13695
E12	37	306	13851
E14	94	294	14932
E15	54	335	14660
E16	184	417	13682
E17	61	122	12801
E18	76	456	7372
E19	76	307	10797
E20	66	417	7982
E21	81	171	6457
E22	104	257	10723

Donor	PfSE	PHA
E1	2	ND
E2	1	429
E3	4	ND
E5	4	151
E6	2	142
E7	5	145
E8	3	122
E9	10	54
E10	4	85
E11	4	177
E12	8	375
E14	3	159
E15	6	273
E16	2	74
E17	2	209
E18	6	97
E19	4	142
E20	6	122
E21	2	79
E22	2	103

c) Day 5

Donor	RBC	PfSE	PHA
E1	66	311	2712
E2	40	130	4000
E3	109	633	2146
E5	119	912	5279
E6	109	679	5184
E7	346	2892	4471
E8	49	469	4797
E9	269	5382	4421
E10	328	3397	2576
E11	226	1578	10180
E12	140	1756	13139
E14	110	1576	11121
E15	103	1850	10342
E16	272	1394	4100
E17	115	1368	3911
E18	344	5873	8380
E19	239	2439	9150
E20	276	4284	5952
E21	ND	1332	5089
E22	235	1209	2280

Donor	PfSE	PHA
E1	5	41
E2	3	100
E3	6	20
E5	8	44
E6	6	48
E7	8	13
E8	10	99
E9	20	16
E10	10	8
E11	7	45
E12	13	94
E14	14	101
E15	18	100
E16	5	15
E17	12	34
E18	17	24
E19	10	38
E20	16	22
E21	ND	ND
E22	5	10

Table A2.2 (continued):

d) Day 7

Donor	RBC	PfSE	PHA
E1	151	1373	1010
E2	54	345	637
E3	336	1851	909
E5	281	5127	714
E6	169	3273	942
E7	532	5770	966
E8	59	1296	1618
E9	245	9809	2112
E10	310	6207	845
E11	397	3335	2395
E12	309	5305	2769
E14	139	6677	1456
E15	202	5584	1169
E16	652	4317	1037
E17	851	2622	1218
E18	440	9115	2345
E19	ND	7654	1110
E20	809	6552	1379
E21	290	5609	1558
E22	374	3828	430

Donor	PfSE	PHA
E1	9	7
E2	6	12
E3	6	3
E5	18	3
E6	19	6
E7	11	2
E8	22	28
E9	40	9
E10	20	3
E11	8	6
E12	17	9
E14	48	10
E15	28	6
E16	7	2
E17	3	1
E18	21	5
E19	ND	ND
E20	8	2
E21	19	5
E22	10	1

e) Day 9

Donor	RBC	PfSE	PHA
E1	315	1399	851
E2	126	652	308
E3	341	1524	691
E5	973	6291	263
E6	679	4177	178
E7	1495	6780	381
E8	152	1683	1080
E9	442	5368	600
E11	1660	2737	747
E12	792	4298	447
E14	251	6313	493
E15	480	6313	553
E18	1038	5361	509
E19	263	4098	580
E20	943	3152	577
E21	100	822	985

Donor	PfSE	PHA
E1	4	3
E2	5	2
E3	4	2
E5	6	0
E6	6	0
E7	5	0
E8	11	7
E9	12	1
E11	2	0
E12	5	1
E14	25	2
E15	13	1
E18	5	0
E19	16	2
E20	3	1
E21	8	10

Table A2.3: Complete lymphoproliferative results for PBMCs of immune individuals by day. Data is given in geometric mean cpm (left-hand tables), and SI (PfSE = PfSE/uRBC, PHA = PHA/CM (CM data not shown), right-hand tables)(ND= not done).

a) Day 3

Donor	RBC	PfSE	PHA
I1	22	36	4609
I2	36	186	11576
I3	12	52	8447
I4	24	60	7031
I5	31	95	13871
I6	37	73	14961
I7	216	345	557
I8	32	71	24152
I9	58	91	34928
I10	55	112	33922
I11	20	49	8528
I12	22	53	3842
I13	71	214	8618
I14	72	274	37243
I15	37	165	29109
I16	80	80	3986
I17	12	335	12624
I18	17	85	33959

Donor	PfSE	PHA
I1	2	355
I2	5	252
I3	4	881
I4	2	347
I5	3	420
I6	2	405
I7	2	2
I8	2	629
I9	2	2071
I10	2	428
I11	2	966
I12	2	186
I13	3	93
I14	4	458
I15	4	821
I16	1	48
I17	27	633
I18	5	1595

b) Day 5

Donor	RBC	PfSE	PHA
I1	10	33	320 .
I2	59	670	2849
I3	48	368	15839
I4	26	703	6003
I5	65	2081	41494
I7	67	1150	15133
I8	ND	338	39717
I11	27	155	25756
I13	214	2738	8000
I14	328	1087	46856
I15	36	1046	34082
I16	70	53	2723
I18	149	557	33125

Donor	PfSE	PHA
I1	3	34
I2	11	89
I3	8	1014
I4	28	224
I5	32	688
I7	17	270
I8	ND	39717
I11	6	3021
I13	13	25
I14	3	340
I15	29	1376
I16	1	75
I18	4	354

Table A2.3 (continued):

c) Day 7

Donor	RBC	PfSE	PHA
I1	16	92	2031
I2	117	11763	13733
I3	146	1413	20
I4	60	7186	31
I5	90	4896	18651
I6	99	294	13533
I7	206	7319	13819
I8	188	1977	9968
I9	481	1153	11974
I10	100	2493	15755
I11	92	439	15420
I12	143	547	12738
I13	1151	7712	5150
I14	442	7816	9893
I15	47	6358	17436
I16	42	51	3251
I17	61	14005	11138
I18	99	1584	2557
I19	79	17095	7379
I20	23	952	3316

Donor	PfSE	PHA
I1	6	348
I2	100	112
I3	10	0
I4	120	1
I5	54	95
I6	3	135
I7	36	82
I8	11	78
I9	2	18
I10	25	419
I11	5	238
I12	4	170
I13	7	38
I14	18	47
I15	134	1378
I16	1	127
I17	229	364
I18	16	16
I19	217	111
I20	42	155

Table A2.4: Complete lymphoproliferative results for PBMCs of naive individuals used as controls in Ghana by day. Data is given in geometric mean cpm (left-hand tables), and SI (PfSE = PfSE/uRBC, PHA = PHA/uRBC, right-hand tables)(ND= not done).

Day 2

Donor	RBC	PfSE	PHA
C2	404	438	2873
C4	24	58	4499

Donor	PfSE	PHA
C2	1	7
C4	2	191

Day 3

Donor	RBC	PfSE	PHA
C2	160	164	3967
C3	28	60	3882
C4	32	114	8257
C5	104	397	13252

Donor	PfSE	PHA
C2	1	25
C3	2	139
C4	4	256
C5	4	129

Day 5

Donor	RBC	PfSE	PHA
C2	73	196	2825
C3	42	361	2741
C4	135	935	7393

Donor	PfSE	PHA
C2	3	39
C3	9	65
C4	7	55

Table A2.4 (continued):

Day 7

Donor	RBC	PfSE	PHA
C2	91	239	1142
C3	65	986	1570
C4	80	1310	3012
C5	38	5561	3203

Donor	PfSE	PHA
C2	3	13
C3	15	24
C4	16	38
C5	146	17

Day 9

Donor	RBC	PfSE	PHA
C2	207	1436	863
C3	98	2186	487
C4	187	1753	1265

Donor	PfSE	PHA
C2	7	4
C3	22	5
C4	9	7

Table A2.5: Kinetic timecourses of lymphoproliferative and IFN-γ responses to PfSE of PBMCs from a) naïve, b) exposed and c) immune individuals (median (PfSE-uRBC) and range for IFN-γ and geometric mean SI (PfSE/uRBC) and 95% CI for lymphoproliferation.

a)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
3	Mean	2.4	2	Mean	6
	CI	1.5, 2.8		Range	0, 382
	n	19		n	19
5	Mean	7.5	4	Mean	32
	CI	4.6, 12.1		Range	5, 1221
	n	19		n	19
7	Mean	16.4	6	Mean	64
	CI	11.4, 23.7		Range	4, 2216
	n	19		n	19
9	Mean	9.2	8	Mean	76
	CI	4.8, 17.7		Range	-0.5, 1072
	n	19		n	18

b)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
2	Mean	1.7	1	Mean	2
	CI	1.5, 2.0		Range	-3, 63
	n	20		n	20
3	Mean	3.6	2	Mean	19
	CI	2.8, 4.6		Range	-2, 1517
	n	20		n	20
5	Mean	9.0	4	Mean	504
	CI	7.0, 11.5		Range	8, 4382
	n	19		n	20
7	Mean	13.5	6	Mean	1089
	CI	4.0, 16.7		Range	13, 4985
	n	19		n	20
9	Mean	6.7	8	Mean	1049
	CI	4.7, 9.6		Range	-4, 5974
	n	16.0		n	19

c)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
3	Mean	2.9	2	Mean	2
	CI	2.0, 4.2		Range	-2, 442
	n	18		n	18
5	Mean	8.2	4	Mean	7
	CI	4.0, 16.7		Range	0.2, 82
	n	12		n	13
7	Mean	18.8	6	Mean	8
	CI	9.0, 39.2		Range	-4, 705
	n	20		n	20

Appendix 3

Table A3.1: Complete IFN-γ (pg/ml) results for PBMCs of naïve individuals by day. Data is given in arithmetic mean (left-hand tables) and malaria-specific responses (PfSE-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	54	ND	ND	94	ND	ND	525	ND	ND
N2	50	ND	ND	144	ND	ND	478	ND	ND
N3	72	ND	77	73	ND	76	84	ND	86
N4	74	ND	82	82	ND	87	965	ND	540
N5	73	ND	78	79	ND	78	164	ND	199
N12	72	ND	ND	73	ND	ND	ND	ND	ND
N13	72	ND	ND	72	ND	ND	ND	ND	ND
N14	72	ND	ND	72	ND	ND	ND	ND	ND
N15	72	ND	ND	74	ND	ND	ND	ND	ND
N16	49	ND	ND	48	ND	ND	ND	ND	ND
N17	47	ND	ND	49	ND	ND	ND	ND	ND
N18	49	ND	ND	60	ND	ND	ND	ND	ND
N19	47	ND	ND	47	ND	ND	ND	ND	ND

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	40	ND	ND
N2	93	ND	ND
N3	1	ND	-1
N4	8	ND	5
N5	5	ND	0
N12	0	ND	ND
N13	0	ND	ND
N14	0	ND	ND
N15	1	ND	ND
N16	-1	ND	ND
N17	1	ND	ND
N18	11	ND	ND
N19	0	ND	ND

b) Day 2

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	55	ND	ND	133	ND	ND	5494	ND	ND
N2	79	ND	ND	461	ND	ND	6122	ND	ND
N3	75	ND	77	100	ND	82	11897	ND	1678
N4	74	ND	83	85	ND	84	12493	ND	1155
N5	73	ND	78	89	ND	79	3379	ND	861
N6	80	84	78	89	86	79	9309	10221	4412
N7	81	94	82	83	99	82	5548	6655	913
N8	73	80	78	114	102	81	7255	9978	2731
N9	75	80	74	78	92	77	601	90	143
N10	77	88	77	83	86	81	3428	590	549
N11	75	89	77	81	79	80	8979	7545	5043
N12	72	71	73	73	71	71	221	611	210
N13	72	74	71	75	74	72	198	756	150
N14	72	73	73	72	72	72	120	292	115
N15	73	71	72	75	72	72	151	297	156
N16	47	47	49	50	48	49	3148	1929	914
N17	48	45	48	48	53	51	3750	1715	163
N18	52	50	48	265	254	258	4397	6317	525
N19	47	48	47	49	47	47	2610	1781	462

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	78	ND	ND
N2	382	ND	ND
N3	25	ND	5
N4	11	ND	1
N5	16	ND	1
N6	9	2	1
N7	2	6	0
N8	41	22	2
N9	3	12	3
N10	7	-1	4
N11	6	-10	4
N12	1	0	-1
N13	3	1	1
N14	0	-1	-1
N15	2	1	0
N16	3	1	0
N17	0	7	3
N18	213	204	210
N19	2	-1	0

c) Day 3

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	ND	ND	ND	5992	ND	ND	377	ND	ND
N2	122	ND	ND	658	ND	ND	3422	ND	ND
N3	73	ND	76	130	ND	83	11163	ND	2030
N4	74	ND	79	114	ND	85	8902	ND	1711
N5	73	ND	77	119	ND	80	5965	ND	3035

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	ND	ND	ND
N2	536	ND	ND
N3	57	ND	7
N4	40	ND	5
N5	46	ND	3

Table A3.1 (continued):

d) Day 4

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	117	ND	ND	1319	ND	ND	7611	ND	ND
N2	90	ND	ND	1311	ND	ND	3764	ND	ND
N3	73	ND	77	298	ND	88	10574	ND	2228
N4	77	ND	81	125	ND	90	9651	ND	1487
N5	73	ND	76	270	ND	83	5358	ND	2797
N6	79	87	78	124	99	83	9662	10419	5443
N7	79	97	80	92	106	83	6803	9124	976
N8	74	87	78	140	150	100	9968	10191	4315
N9	74	78	78	86	99	95	4158	586	518
N10	76	90	76	109	116	101	6575	1000	775
N11	78	86	76	84	120	81	8276	6104	2801
N12	72	73	71	106	75	74	641	1782	309
N13	71	74	72	80	77	72	553	533	128
N14	75	72	72	80	79	77	1090	4993	243
N15	73	72	71	83	84	77	486	1502	312
N16	48	47	47	64	52	49	5074	6247	376
N17	48	46	47	53	59	49	2323	3890	337
N18	50	53	49	480	727	233	5963	6890	1623
N19	47	46	47	61	55	52	1223	872	353

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	1202	ND	ND
N2	1221	ND	ND
N3	225	ND	11
N4	48	ND	9
N5	197	ND	7
N6	45	12	5
N7	13	9	4
N8	66	63	22
N9	12	21	17
N10	32	26	25
N11	6	34	5
N12	35	3	2
N13	9	3	1
N14	6	7	5
N15	10	11	6
N16	16	5	2
N17	5	13	2
N18	429	675	185
N19	14	9	6

e) Day 5

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	ND	ND	ND	1923	ND	ND	ND	ND	ND
N2	104	ND	ND	669	ND	ND	1794	ND	ND
N3	72	ND	77	349	ND	98	9045	ND	1309
N4	73	ND	81	194	ND	90	6165	ND	983
N5	72	ND	76	492	ND	86	2521	ND	1004

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	ND	ND	ND
N2	565	ND	ND
N3	277	ND	22
N4	121	ND	9
N5	420	ND	9

f) Day 6

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	80	ND	ND	2297	ND	ND	ND	ND	ND
N2	96	ND	ND	1058	ND	ND	3721	ND	ND
N3	72	ND	78	397	ND	116	9795	ND	1302
N4	75	ND	79	302	ND	109	7912	ND	541
N5	72	ND	77	366	ND	98	2545	ND	1184
N6	83	112	77	211	129	84	10945	10662	5940
N7	79	97	81	137	157	91	6159	7561	563
N8	75	102	77	196	168	92	8128	8925	4438
N9	74	81	77	90	127	98	4350	386	434
N10	76	86	75	768	588	160	4533	1904	364
N11	75	146	75	104	93	110	8055	5893	2656
N12	74	72	71	101	75	79	261	3329	202
N13	71	71	72	75	87	73	171	425	135
N14	73	72	73	124	108	115	610	1510	150
N15	74	73	71	125	80	76	345	1172	227
N16	48	45	48	105	62	60	5136	5805	231
N17	48	46	46	65	102	56	663	1553	165
N18	49	52	48	1185	817	147	5174	5770	834
N19	51	45	48	114	190	85	3610	3833	211

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	2216	ND	ND
N2	962	ND	ND
N3	325	ND	38
N4	226	ND	30
N5	294	ND	21
N6	128	17	7
N7	58	60	10
N8	122	66	15
N9	16	47	20
N10	692	502	86
N11	29	-54	35
N12	27	3	8
N13	3	16	2
N14	51	36	42
N15	51	8	5
N16	57	17	13
N17	17	56	10
N18	1136	765	99
N19	64	144	37

g) Day 7

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	60	ND	ND	2626	ND	ND	4471	ND	ND
N2	49	ND	ND	2453	ND	ND	2148	ND	ND
N3	74	ND	77	345	ND	98	9161	ND	819
N4	75	ND	79	408	ND	139	7206	ND	611
N5	74	ND	76	221	ND	116	2195	ND	1854

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	2566	ND	ND
N2	2404	ND	ND
N3	272	ND	22
N4	332	ND	60
N5	146	ND	41

Table A3.1 (continued):

h) Day 8

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	74	ND	ND	ND	ND	ND	6561	ND	ND
N2	61	ND	ND	1134	ND	ND	3721	ND	ND
N3	74	ND	76	408	ND	83	12466	ND	1144
N4	74	ND	81	145	ND	145	8523	ND	1023
N5	73	ND	77	244	ND	102	3640	ND	1783
N6	77	100	79	119	111	84	11497	8590	3910
N7	78	127	78	206	181	115	5799	7508	1035
N8	75	118	77	193	141	109	10079	9470	1773
N9	75	95	74	105	154	99	3122	387	287
N10	75	81	76	320	427	117	849	123	219
N11	76	105	74	158	138	119	8293	3122	1251
N12	72	71	71	71	71	71	72	74	72
N13	71	71	73	72	71	71	75	75	72
N14	73	73	72	116	107	151	551	3524	199
N15	74	73	71	96	83	81	209	1162	244
N16	47	46	48	108	50	60	4275	4171	371
N17	47	46	46	86	67	66	1958	4187	191
N18	51	57	47	502	689	451	6324	4583	345
N19	49	46	48	146	83	134	1271	4957	199

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	ND	ND	ND
N2	1072	ND	ND
N3	334	ND	7
N4	71	ND	64
N5	171	ND	26
N6	41	10	4
N7	128	53	36
N8	118	23	32
N9	30	59	25
N10	245	346	41
N11	82	33	45
N12	0	0	0
N13	0	0	-1
N14	43	33	79
N15	22	11	10
N16	61	4	12
N17	39	21	20
N18	451	632	404
N19	96	36	87

Table A3.2: Complete IFN-γ (pg/ml) results for PBMCs of exposed individuals by day. Data is given in arithmetic mean (left-hand tables) and malaria-specific responses (PfSE-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	51	48	56	49	48	52	ND	93	70
E2	49	48	59	52	48	51	85	123	92
E3	53	53	51	52	54	50	ND	240	123
E5	48	49	52	49	48	51	69	67	61
E6	70	49	67	121	51	54	195	385	276
E7	48	47	51	48	47	43	51	60	53
E8	53	52	53	55	55	53	380	300	145
E9	37	46	46	101	138	131	626	868	531
E10	50	50	55	56	51	57	68	80	71
E11	36	35	39	37	39	41	837	735	328
E12	36	35	40	43	41	39	494	992	508
E14	32	31	37	38	37	40	54	69	54
E15	34	35	37	37	39	40	166	164	85
E16	65	65	59	66	67	72	205	281	236
E17	47	48	54	47	47	53	68	87	82
E18	34	37	41	37	40	39	48	73	50
E19	59	56	59	59	57	52	497	506	220
E20	37	75	46	46	43	47	52	72	55
E21	50	58	56	65	65	64	335	337	317
E22	50	48	52	47	50	52	95	71	102

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	-3	0	-4
E2	3	0	-8
E3	-1	1	-1
E5	1	-1	-1
E6	51	3	-12
E7	0	1	-8
E8	2	4	0
E9	63	92	86
E10	6	1	2
E11	1	4	2
E12	7	6	0
E14	6	5	3
E15	3	4	3
E16	0	2	13
E17	1	0	-1
E18	4	3	-2
E19	0	1	-7
E20	9	-32	2
E21	15	7	9
E22	-3	2	-1

Table A3.2 (continued):

b) Day 2

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	57	50	51	55	51	57	ND	ND	273
E2	48	51	55	46	56	54	380	379	141
E3	53	52	51	70	82	96	ND	800	152
E5	50	48	51	49	51	51	274	683	95
E6	52	58	59	77	92	69	1592	2269	1844
E7	46	49	52	56	38	36	223	192	82
E8	55	77	58	96	235	66	3134	5349	454
E9	48	92	53	1564	880	263	2672	4768	2068
E10	63	76	63	327	175	78	899	475	248
E11	38	36	39	49	44	40	3035	5490	820
E12	35	37	38	51	50	45	4781	4391	2773
E14	32	35	36	51	45	41	694	1018	117
E15	34	37	37	53	52	43	1925	3064	503
E16	73	82	62	193	211	93	3076	4397	878
E17	49	55	53	51	51	58	488	710	504
E18	35	41	44	73	117	58	521	457	99
E19	54	62	62	159	120	66	3543	3807	703
E20	42	38	60	81	53	52	193	1032	89
E21	77	62	71	141	119	89	1381	2062	672
E22	53	64	53	65	96	58	2703	3730	603

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	-2	1	6
E2	-1	5	-1
E3	17	31	45
E5	-1	3	-1
E6	25	34	10
E7	10	-10	-16
E8	41	158	8
E9	1517	788	210
E10	265	99	16
E11	11	8	1
E12	16	13	6
E14	19	11	5
E15	19	16	6
E16	120	129	31
E17	1	-4	5
E18	38	76	14
E19	105	58	4
E20	40	15	-8
E21	64	57	18
E22	12	32	4

c) Day 4

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	58	57	54	97	116	56	889	773	95
E2	48	47	50	55	58	53	5055	2253	267
E3	54	68	50	858	650	69	2625	3226	221
E5	47	48	52	306	140	106	1689	1553	137
E6	69	86	57	566	332	88	5034	3752	2191
E7	50	58	53	182	198	81	310	226	126
E8	53	58	55	649	852	149	5848	6799	429
E9	62	146	63	4444	2744	309	6917	4415	2210
E10	177	351	70	3268	2813	863	1883	1144	223
E11	43	39	40	65	61	44	6303	3240	1687
E12	36	38	38	288	127	62	4625	7227	1481
E14	32	34	36	277	624	80	1078	4159	153
E15	35	37	39	387	217	79	3101	2651	624
E16	133	147	86	1270	2000	337	9117	10315	2124
E17	54	147	65	1979	471	87	1588	2914	603
E18	37	41	39	1180	802	178	2084	2647	91
E19	68	476	54	3961	2632	351	8997	7688	1512
E20	52	47	53	3840	1160	560	818	729	174
E21	ND	321	67	3287	4020	655	5200	5308	1262
E22	67	78	64	571	524	144	4733	5555	746

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	39	59	2
E2	8	11	3
E3	804	582	19
E5	259	92	54
E6	496	246	31
E7	132	140	28
E8	596	794	94
E9	4382	2598	246
E10	3091	2461	792
E11	23	22	4
E12	253	89	25
E14	246	590	43
E15	353	180	40
E16	1137	1852	251
E17	1925	324	23
E18	1143	761	139
E19	3894	2156	297
E20	3788	1113	508
E21	ND	3699	588
E22	504	446	80

Table A3.2 (continued):

d) Day 6

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	49	57	46	120	58	466	2321	519	70
E2	52	49	51	65	95	60	4827	4888	212
E3	131	135	53	1100	134	2475	3905	3520	145
E5	48	49	51	770	602	129	460	1613	90
E6	96	111	56	695	924	151	5604	4874	1464
E7	57	93	56	1411	295	80	519	403	67
E8	54	55	64	1198	1218	101	4601	6364	377
E9	51	123	54	5035	2718	508	5062	7806	1106
E10	405	557	88	3675	3961	1524	3113	3081	236
E11	41	89	37	87	116	58	4807	6430	1346
E12	62	55	39	441	715	115	5511	5353	1678
E14	32	34	36	2024	2412	331	2972	1370	110
E15	35	38	36	1070	1223	179	3101	3565	482
E16	776	797	143	3287	3643	1716	10702	7882	1407
E17	146	236	93	1375	665	599	1512	3063	465
E18	42	51	37	1005	1692	321	3322	2428	75
E19	ND	ND	ND	8521	5269	650	8189	8984	551
E20	ND	46	41	1844	4055	840	692	2365	173
E21	142	ND	75	4443	7929	990	5124	4775	841
E22	81	225	56	1542	1747	721	4308	3881	745

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	70	1	420
E2	13	46	9
E3	970	-1	2422
E5	723	553	77
E6	599	813	95
E7	1354	202	24
E8	1144	1163	37
E9	4985	2596	454
E10	3270	3404	1435
E11	46	27	20
E12	379	660	76
E14	1992	2379	295
E15	1034	1185	142
E16	2511	2846	1573
E17	1228	429	506
E18	963	1641	284
E19	ND	ND	ND
E20	ND	4009	799
E21	4301	ND	916
E22	1461	1522	665

e) Day 8

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	54	51	49	123	64	178	609	219	63
E2	49	56	49	95	77	56	3923	5089	140
E3	100	240	57	1163	108	2103	3838	2799	203
E5	67	57	51	1040	1208	464	1169	2015	102
E6	181	162	58	1018	742	318	4917	5806	1828
E7	54	73	57	1188	165	137	1717	425	82
E8	56	58	52	3171	1484	103	4654	4776	467
E9	98	169	66	3244	3541	900	7481	6353	1443
E10	405	295	85	4384	3253	1637	2303	1383	163
E11	101	58	42	96	166	46	5921	5247	1301
E12	57	74	42	913	442	103	3485	7392	1869
E14	32	47	37	2561	3403	732	1721	1558	127
E15	37	75	37	692	651	328	3764	1602	468
E16	ND	ND	245	3031	3036	1925	7270	7553	1667
E17	207	218	83	1257	1032	919	2306	1528	315
E18	50	54	42	457	568	237	1650	3358	61
E19	192	141	54	6166	6977	145	5814	5953	520
E20	72	84	63	923	1692	1950	1887	1133	151
E21	74	104	59	4824	4476	131	4214	8274	839
E22	97	296	56	1883	2354	256	1621	5629	555

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	69	13	129
E2	46	20	6
E3	1064	-132	2046
E5	973	1151	413
E6	837	580	260
E7	1134	91	81
E8	3115	1426	51
E9	3146	3372	834
E10	3979	2958	1553
E11	-4	108	4
E12	857	369	61
E14	2529	3356	695
E15	655	576	290
E16	ND	ND	1679
E17	1049	814	836
E18	407	513	195
E19	5974	6835	92
E20	851	1608	1887
E21	4750	4371	72
E22	1786	2058	200

Table A3.3: Complete IFN-γ (pg/ml) results for PBMCs of immune individuals by day. Data is given in arithmetic mean (left-hand tables) and malaria-specific responses (PfSE-uRBC, right-hand tables)(ND = not done).

a) Day 2

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	59	58	57	60	58	57	56	92	178
I2	60	56	61	57	73	67	56	1174	974
I3	56	59	59	56	60	60	56	606	193
I4	56	57	57	57	60	59	59	160	57
I5	56	59	56	58	60	59	57	268	211
I6	58	58	57	56	57	58	56	196	62
I7	59	56	58	56	61	61	56	593	2864
I8	58	58	61	54	56	57	56	1378	103
I9	56	57	56	54	55	55	56	1699	177
I10	57	60	59	55	57	62	56	425	115
I11	57	56	56	56	58	57	57	1211	73
I12	57	58	57	58	61	64	57	881	1055
I13	58	58	61	57	64	60	58	189	113
I14	56	57	58	57	66	63	59	5506	85
I15	57	59	58	58	65	68	66	2380	119
I16	57	56	56	57	56	56	59	60	58
I17	63	58	66	67	500	290	104	3933	503
I18	57	57	59	55	63	60	56	8433	296

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	1	0	-4
I2	17	6	-1
I3	1	1	0
I4	3	2	1
I5	1	3	-1
I6	0	0	-1
I7	5	2	0
I8	-2	-4	1
I9	-1	-1	2
I10	-2	3	1
I11	2	0	1
I12	3	7	-2
I13	6	-1	1
I14	8	4	2
I15	6	10	8
I16	0	0	2
I17	442	224	38
I18	6	1	2

b) Day 4

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	59	58	58	59	59	59	63	61	2043
I2	57	57	72	57	139	157	64	2482	4275
I3	55	57	58	55	60	59	56	543	1823
I4	57	58	56	58	69	64	63	169	57
I5	56	58	56	56	87	76	65	1746	4248
I7	57	57	62	56	64	72	58	639	3555
I8	57	57	58	54	58	58	54	2991	338
I11	57	56	57	57	56	57	57	4580	170
I13	57	59	62	58	66	63	59	219	467
I14	56	57	58	60	91	79	73	10953	98
I15	57	57	58	57	74	79	62	4002	211
I16	56	55	56	56	56	55	57	59	57
I18	57	57	61	56	61	60	56	5236	280

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	0	1	4
I2	82	85	7
I3	3	1	1
I4	12	9	5
I5	29	19	9
I7	7	10	2
I8	1	1	0
I11	0	0	0
I13	7	2	1
I14	33	21	13
I15	17	21	5
I16	0	-1	1
I18	4	-2	0

Table A3.3 (continued):

c) Day 6

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	58	57	58	58	59	59	57	74	1971
I2	56	58	60	68	116	107	63	2903	3652
I3	57	55	57	57	59	61	59	1699	3920
I4	56	58	56	58	69	67	62	155	72
I5	64	56	58	56	99	69	57	1124	3622
I6	58	59	59	57	58	56	55	332	74
I7	55	56	66	56	64	58	58	561	2763
I8	57	60	56	55	58	55	56	2185	405
I9	62	57	57	54	57	58	55	1206	405
I10	57	57	58	55	64	59	58	1646	1765
I11	58	56	57	55	59	58	57	5738	89
I12	59	63	57	58	59	61	57	1314	5621
I13	56	63	63	58	88	72	74	409	460
I14	56	58	59	57	151	116	94	3426	292
I15	57	58	57	56	131	112	62	3022	1152
I16	56	56	56	56	56	55	59	61	57
I17	64	56	88	58	528	424	104	3837	14627
I18	57	57	59	56	70	62	59	7445	372
I19	57	58	87	69	763	1143	136	7694	7561
I20	57	57	61	63	66	60	59	131	484

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	2	2	-1
I2	59	47	-6
I3	5	5	2
I4	11	11	4
I5	43	11	1
I6	-1	-3	-1
I7	8	-7	2
I8	-1	0	1
I9	0	1	1
I10	7	1	3
I11	3	1	2
I12	-4	3	-1
I13	26	9	15
I14	93	57	37
I15	74	55	6
I16	0	0	3
I17	471	336	46
I18	13	3	3
I19	705	1056	67
I20	9	-1	-4

Table A3.4: Complete IFN-γ (pg/ml) results for PBMCs of naive individuals used as controls in Ghana by day. Data is given in arithmetic mean (left-hand tables) and malaria-specific responses (PfSE-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	65	80	70	119	69	59	214	1040	202
C4	53	50	52	52	52	53	200	170	122

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	54	-11	-10
C4	-2	3	1

b) Day 2

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	63	71	53	70	89	78	507	4085	300
C3	57	51	57	51	51	62	92	303	140
C4	57	54	60	53	57	54	1086	2348	464
C5	58	58	56	69	65	63	94	ND	ND

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	7	17	24
C3	-5	1	5
C4	-5	3	-6
C5	11	8	7

c) Day 4

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	60	70	61	187	69	70	1126	4314	281
C3	51	50	53	76	68	58	88	898	169
C4	52	49	59	168	135	79	5967	4954	1137

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	126	0	8
C3	24	18	4
C4	116	86	20

d) Day 6

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	81	68	74	229	513	70	1539	4061	231
C3	61	55	52	72	120	63	133	884	189
C4	50	50	47	381	131	74	2314	5877	982
C5	60	56	57	111	103	73	121	ND	ND

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	147	445	-4
C3	11	65	11
C4	331	81	26
C5	51	47	17

e) Day 8

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	97	86	65	416	1233	77	872	3198	137
C3	48	62	52	124	78	68	113	284	212
C4	51	52	47	508	151	80	2550	5263	817

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	320	1147	12
C3	75	17	16
C4	457	99	33

Table A3.5: Kinetic timecourses of lymphoproliferative and IFN-γ responses to PfSE of PBMCs from a) naïve, b) exposed and c) immune individuals (median (PfSE-uRBC) and range for IFN-γ (pg/ml) and geometric mean SI (PfSE/uRBC) and 95% CI for lymphoproliferation).

a)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
3	Mean	2.4	2	Mean	6
	CI	1.5, 2.8		Range	0, 382
	n	19		n	19
5	Mean	7.5	4	Mean	32
	CI	4.6, 12.1		Range	5, 1221
	n	19		n	19
7	Mean	16.4	6	Mean	64
	CI	11.4, 23.7		Range	4, 2216
	n	19		n	19
9	Mean	9.2	8	Mean	76
	CI	4.8, 17.7		Range	-0.5, 1072
	n	19		n	18

b)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
2	Mean	1.7	1	Mean	2
	CI	1.5, 2.0		Range	-3, 63
	n	20		n	20
3	Mean	3.6	2	Mean	19
	CI	2.8, 4.6		Range	-2, 1517
	n	20		n	20
5	Mean	9.0	4	Mean	504
	CI	7.0, 11.5		Range	8, 4382
	n	19		n	20
7	Mean	13.5	6	Mean	1089
	CI	4.0, 16.7		Range	13, 4985
	n	19		n	20
9	Mean	6.7	8	Mean	1049
	CI	4.7, 9.6		Range	-4, 5974
	n	16.0		n	19

c)

Proliferation data			IFN-γ data		
Day	Stats	SI	Day	Stats	IFN-γ (pg/ml)
3	Mean	2.9	2	Mean	2
	CI	2.0, 4.2		Range	-2, 442
	n	18		n	18
5	Mean	8.2	4	Mean	7
	CI	4.0, 16.7		Range	0.2, 82
	n	12		n	13
7	Mean	18.8	6	Mean	8
	CI	9.0, 39.2		Range	-4, 705
	n	20		n	20

Table A3.6: IL-12 p70 (pg/ml) results for donors a) N3, b) N4 and c) N5 (- indicates that free IL-12 p70 in cell culture supernatants was below LLD = 107 pg/ml).

a) N2

Hours	RBC	PfSE 1:320	PfSE 1:640	PfSE 1:1280	PfSE 1:2560	LPS+ IFN-γ
4	-	-	-	-	-	-
20	-	-	-	-	-	-
28	-	-	-	-	-	-
45	113	-	-	-	-	-
51	131	-	-	-	-	-
68	110	-	-	-	-	-
76	-	-	-	-	-	107
92	-	-	-	-	-	-
100	-	-	-	-	-	-
115	-	-	-	-	-	-

b) N3

Hours	RBC	PfSE 1:320	PfSE 1:640	PfSE 1:1280	PfSE 1:2560	LPS+ IFN-γ
4	-	-	-	-	-	113
20	-	-	-	-	-	143
28	-	-	-	-	-	155
45	-	-	-	-	108	155
51	-	-	-	-	107	142
68	-	-	-	-	-	139
76	-	-	-	-	-	139
92	-	-	-	-	-	150
100	-	-	-	-	114	141
115	-	-	-	-	120	131

c) N5

Hours	RBC	PfSE 1:320	PfSE 1:640	PfSE 1:1280	PfSE 1:2560	LPS+ IFN-γ
4	-	-	-	-	112	-
20	-	-	-	-	-	107
28	-	-	-	-	-	-
45	-	-	-	-	-	109
51	-	-	-	-	-	-
68	-	-	-	-	109	-
76	-	-	-	-	-	-
92	-	-	-	-	-	108
100	-	-	-	-	-	-
115	-	-	-	107	-	100

Table A3.7: Complete IL-12 p40 (pg/ml) results for PBMCs of a) naïve, b) exposed, c) immune and d) control (naïve) individuals on day 2. Data is given in arithmetic mean (left-hand tables) and malaria-specific responses (PfSE-uRBC, right-hand tables).

a)

Donor	RBC	PfSE
N2	167	147
N3	213	213
N4	378	364
N5	204	199
N6	320	305
N7	153	156
N8	147	145
N9	186	207
N10	224	232
N11	184	212
N12	199	195
N13	214	225
N14	211	223
N15	170	172
N16	161	173
N17	175	163
N18	193	177
N19	176	176

Donor	PfSE
N2	-20
N3	0
N4	-14
N5	-5
N6	-15
N7	3
N8	-2
N9	21
N10	8
N11	28
N12	-4
N13	11
N14	12
N15	2
N16	11
N17	-12
N18	-16
N19	0

b)

Donor	RBC	PfSE
E1	187	190
E2	207	221
E3	210	219
E5	326	351
E6	372	388
E7	173	153
E8	205	194
E9	1124	1654
E10	259	285
E11	562	592
E12	776	870
E14	279	372
E15	202	223
E16	245	243
E17	201	169
E18	267	258
E19	173	200
E20	138	132
E21	173	230
E22	184	171

Donor	PfSE
E1	3
E2	14
E3	10
E5	25
E6	16
E7	-20
E8	-11
E9	530
E10	26
E11	30
E12	94
E14	93
E15	21
E16	-2
E17	-32
E18	-9
E19	26
E20	-5
E21	57
E22	-12

Table A3.7 (continued):

c)

Donor	RBC	PfSE
I1	155	171
I2	173	200
I3	168	185
I4	184	183
I5	351	340
I6	806	261
I7	203	199
I8	183	148
I9	249	322
I10	191	220
I11	189	198
I12	182	181
I13	212	250
I14	384	379
I15	192	193
I16	179	214
I17	185	193
I18	224	264
I19	187	200
I20	162	178

Donor	PfSE
I1	16
I2	26
I3	17
I4	-1
I5	-11
I6	-545
I7	-4
I8	-34
I9	73
I10	30
I11	8
I12	-2
I13	38
I14	-5
I15	1
I16	34
I17	8
I18	40
I19	14
I20	16

d)

Donor	RBC	PfSE
C2	138	127
C3	145	135
C4	156	152
C5	185	174

Donor	PfSE
C2	-12
C3	-10
C4	-4
C5	-11

Table A3.8: Geometric mean SI and 95% CI of control IgG/PfSE-stimulated and αIL-12/PfSE-stimulated PBMCs from a) naïve, b) exposed and c) immune individuals for each day.

a)

	PfSE+IgG		PfSE+αIL-12				
Day	Mean	CI	Mean	CI	t	p	n
3	1.7	1.2, 2.3	1.9	1.4, 2.6	1.15	>0.05	13
5	3.3	1.7, 6.4	4.8	2.7, 8.7	1.70	>0.05	14
7	6.8	3.3, 14.0	9.1	5.5, 15.2	1.49	>0.05	14
9	3.4	1.6, 7.2	8.1	4.3, 15.5	3.61	0.003	14

b)

	PfSE+IgG		PfSE+αIL-12				
Day	Mean	CI	Mean	CI	t	p	n
2	1.4	1.2, 1.7	1.4	1.1, 1.7	0.46	>0.05	20
3	3.0	2.4, 3.7	3.2	2.6, 4.0	0.75	>0.05	20
5	6.5	4.9, 8.5	7.0	5.2, 9.4	0.93	>0.05	19
7	10.1	7.2, 14.3	14.1	9.8, 20.3	2.79	0.013	18
9	5.6	4.0, 7.7	8.2	4.9, 13.5	3.11	0.007	16

c)

	PfSE+IgG		PfSE+αIL-12				
Day	Mean	CI	Mean	CI	t	p	n
3	1.8	1.3, 2.7	2.0	1.2, 3.5	0.45	>0.05	18
5	2.1	0.6, 7.5	2.7	1.0, 7.4	0.92	>0.05	11
7	5.5	3.2, 9.5	3.1	1.9, 4.8	2.52	0.021	20

Table A3.9: Complete lymphoproliferative results for PBMCs of naïve individuals by day. Data is given in geometric mean cpm (left-hand tables) and SI (PfSE/uRBC with and without control IgG or αIL-12, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	398	ND	ND	331	ND	ND	547	ND	ND
N2	303	ND	ND	174	ND	ND	470	ND	ND
N3	667	ND	684	600	ND	758	794	ND	560
N4	908	ND	963	830	ND	593	1055	ND	1128
N5	600	ND	1203	489	ND	562	1606	ND	1075

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	1	ND	ND
N2	1	ND	ND
N3	1	ND	1
N4	1	ND	1
N5	1	ND	0

b) Day 2

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	431	ND	ND	794	ND	ND	72158	ND	ND
N2	221	ND	ND	491	ND	ND	71656	ND	ND
N3	730	ND	886	1048	ND	1102	38149	ND	32675
N4	1102	ND	1043	1101	ND	953	59626	ND	53680
N5	359	ND	432	466	ND	480	32188	ND	25513

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	2	ND	ND
N2	2	ND	ND
N3	1	ND	1
N4	1	ND	1
N5	1	ND	1

c) Day 3

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	89	ND	ND	2282	ND	ND	41677	ND	ND
N2	86	ND	ND	2519	ND	ND	31224	ND	ND
N3	1093	ND	1028	1606	ND	1407	72535	ND	69616
N4	925	ND	823	1274	ND	1150	92917	ND	86822
N5	479	ND	497	1934	ND	787	91055	ND	65940
N6	794	805	662	911	686	754	40866	44511	41080
N7	576	723	500	642	830	751	55953	49490	48538
N8	488	693	512	1252	1029	972	38725	41945	37843
N9	515	526	390	853	1023	526	41170	7143	25362
N10	498	448	509	717	577	677	57674	21148	35696
N11	561	449	419	740	381	893	47383	26981	33108
N12	406	451	359	805	941	773	58313	57316	35041
N13	246	255	221	375	313	438	16725	18019	17761
N14	144	159	163	187	226	150	24496	34061	29497
N15	233	ND	216	318	257	225	18379	22072	19902
N16	47	74	46	111	167	83	14449	15266	13088
N17	39	34	24	50	87	51	19073	22533	18085
N18	85	103	88	394	504	542	19200	16073	14727
N19	22	28	19	84	79	69	27230	27256	29790

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	26	ND	ND
N2	29	ND	ND
N3	1	ND	1
N4	1	ND	1
N5	4	ND	2
N6	1	1	1
N7	1	1	2
N8	3	1	2
N9	2	2	1
N10	1	1	1
N11	1	1	2
N12	2	2	2
N13	2	1	2
N14	1	1	1
N15	1	ND	1
N16	2	2	2
N17	1	3	2
N18	5	5	6
N19	4	3	4

d) Day 4

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	414	ND	ND	5592	ND	ND	93653	ND	ND
N2	164	ND	ND	7249	ND	ND	104946	ND	ND
N3	1226	ND	1319	3083	ND	1757	96277	ND	90584
N4	930	ND	1068	2388	ND	1409	96082	ND	77284
N5	648	ND	655	3061	ND	983	72597	ND	72778

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	14	ND	ND
N2	44	ND	ND
N3	3	ND	1
N4	3	ND	1
N5	5	ND	2

Table A3.9 (continued):

e) Day 5

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	554	ND	ND	11151	ND	ND	ND	ND	ND
N2	372	ND	ND	21461	ND	ND	90289	ND	ND
N3	1347	ND	1408	3881	ND	2272	107932	ND	78674
N4	731	ND	602	2170	ND	1359	58922	ND	42786
N5	712	ND	590	4508	ND	1465	53713	ND	53977
N6	988	1525	1274	2473	1910	1796	26546	27437	19236
N7	738	1307	791	3071	2524	1674	18139	21338	13714
N8	1005	1689	1479	4388	4109	3912	34026	33695	28714
N9	685	1703	1580	4516	3568	5082	33719	8905	19289
N10	745	800	792	2758	1970	2803	21438	5041	13421
N11	668	829	795	4052	4542	2030	41493	13392	17508
N12	616	1707	1164	11322	5100	5323	17051	28222	18198
N13	543	513	481	1346	2049	955	10303	11515	9816
N14	108	105	67	324	231	313	33284	47017	32887
N15	48	242	63	477	662	263	10410	17035	13003
N16	62	205	60	317	59	270	4427	5789	2568
N17	28	25	20	361	575	449	10603	10987	12144
N18	36	120	60	2036	1910	2013	7414	6256	5363
N19	50	50	38	940	832	1098	6505	5989	4938

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	20	ND	ND
N2	58	ND	ND
N3	3	ND	2
N4	3	ND	2
N5	6	ND	2
N6	3	1	1
N7	4	2	2
N8	4	2	3
N9	7	2	3
N10	4	2	4
N11	6	5	3
N12	18	3	5
N13	2	4	2
N14	3	2	5
N15	10	3	4
N16	5	0	5
N17	13	23	22
N18	56	16	33
N19	19	17	29

f) Day 6

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	2183	ND	ND	27706	ND	ND	30434	ND	ND
N2	632	ND	ND	58520	ND	ND	49461	ND	ND
N3	1296	ND	1577	4980	ND	2802	47210	ND	25114
N4	1086	ND	670	1627	ND	2146	16739	ND	9124
N5	1119	ND	1167	14313	ND	4167	27989	ND	18189

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	13	ND	ND
N2	93	ND	ND
N3	4	ND	2
N4	1	ND	3
N5	13	ND	4

g) Day 7

Donor	RBC	RBC+ IgG	RBC+ aIL-12	PfSE	PfSE+ IgG	PfSE+ aIL-12	PHA	PHA+ IgG	PHA+ aIL-12
N1	2077	ND	ND	36539	ND	ND	11494	ND	ND
N2	797	ND	ND	49436	ND	ND	18652	ND	ND
N3	3081	ND	3394	14536	ND	6275	21516	ND	18262
N4	1279	ND	1479	13961	ND	1959	11928	ND	7066
N5	1489	ND	1752	22477	ND	4800	13941	ND	9275
N6	1888	4854	1327	10924	4307	2972	6813	8765	7505
N7	941	2880	1366	7339	10077	6891	3363	3901	4828
N8	1316	4720	1504	11270	6560	8092	7821	9749	9571
N9	1117	2239	3006	12160	14009	14547	7818	3764	5375
N10	1415	1584	1236	14039	18811	13090	6258	1963	4402
N11	1012	3214	992	23123	10689	17276	11285	8496	6928
N12	749	5917	5139	8925	7548	9111	1338	2581	2024
N13	186	287	305	1897	5509	11321	1927	2540	3497
N14	254	368	335	4846	4696	3738	14967	14184	16821
N15	141	549	203	4513	2671	1554	6542	7258	7311
N16	142	349	309	11348	4952	6656	1984	2551	2865
N17	244	263	253	6863	10374	3737	10794	11068	10367
N18	146	404	235	4566	4516	3036	2708	2114	4271
N19	437	303	484	12457	14426	11122	2900	2313	4030

Donor	PfSE	PfSE+ IgG	PfSE+ aIL-12
N1	18	ND	ND
N2	62	ND	ND
N3	5	ND	2
N4	11	ND	1
N5	15	ND	3
N6	6	1	2
N7	8	3	5
N8	9	1	5
N9	11	6	5
N10	10	12	11
N11	23	3	17
N12	12	1	2
N13	10	19	37
N14	19	13	11
N15	32	5	8
N16	80	14	22
N17	28	39	15
N18	31	11	13
N19	28	48	23

Table A3.9 (continued):

h) Day 9

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
N1	1610	ND	ND	17154	ND	ND	1916	ND	ND
N2	536	ND	ND	26635	ND	ND	3439	ND	ND
N3	3077	ND	4390	13394	ND	3148	10386	ND	5949
N4	1678	ND	2882	15273	ND	3537	7033	ND	3487
N5	1864	ND	1632	10862	ND	6258	4162	ND	3738
N6	5567	5065	5927	9197	8721	9370	11343	11337	10495
N7	1563	17447	3996	45663	49521	55488	5012	3643	8796
N8	2308	12137	7201	55774	27124	12394	8834	9491	7599
N9	1889	6190	3748	18343	35022	31330	4114	4398	5459
N10	5309	3938	3770	23834	31131	26190	7007	3265	5378
N11	3165	6979	2709	44769	23305	26760	10712	6104	5215
N12	406	237	43	159	85	156	166	266	384
N13	474	814	419	566	647	773	458	418	550
N14	322	676	334	3989	7179	8589	4368	4560	5986
N15	475	934	195	4420	3397	2817	3490	3980	3233
N16	35	225	160	3619	531	3816	683	414	766
N17	125	65	82	5779	3644	5962	3571	3348	4411
N18	574	2584	372	3027	2552	1888	1672	1696	3625
N19	421	618	1034	7212	8381	12248	1835	1154	1903

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
N1	11	ND	ND
N2	50	ND	ND
N3	4	ND	1
N4	9	ND	1
N5	6	ND	4
N6	2	2	2
N7	29	3	14
N8	24	2	2
N9	10	6	8
N10	4	8	7
N11	14	3	10
N12	0	0	4
N13	1	1	2
N14	12	11	26
N15	9	4	14
N16	105	2	24
N17	46	56	73
N18	5	1	5
N19	17	14	12

Table A3.10: Complete lymphoproliferative results for PBMCs of exposed individuals by day. Data is given in geometric mean cpm (left-hand tables) and SI (PfSE/uRBC with and without control IgG or αIL-12, right-hand tables)(ND = not done).

a) Day 2

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	19	20	14	25	18	19	ND	2174	2257
E2	19	30	32	27	24	15	2017	2871	3759
E3	15	26	20	32	34	37	ND	2180	2210
E5	38	52	52	57	42	56	1146	1336	935
E6	28	19	25	26	23	21	578	536	595
E7	29	40	37	66	53	62	4204	3862	3679
E8	15	19	32	45	24	18	2908	2908	2934
E9	81	85	72	164	146	132	2318	2513	2618
E10	48	50	53	86	81	90	3104	3266	3063
E11	99	76	80	115	103	89	4225	4489	4103
E12	24	26	28	60	82	103	2888	3282	3068
E14	61	52	51	76	69	66	6062	5936	6325
E15	26	23	35	64	55	42	4636	4949	4798
E16	39	44	50	58	57	60	4620	4291	4256
E17	23	25	31	35	37	29	1915	1387	1729
E18	29	30	21	68	44	53	3888	3443	3225
E19	28	31	43	65	59	109	1714	1470	1726
E20	41	60	47	74	93	82	4854	4718	4541
E21	21	22	23	21	34	43	841	762	758
E22	22	22	27	43	39	36	2258	2423	2335

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	1	1	1
E2	1	1	0
E3	2	1	2
E5	2	1	1
E6	1	1	1
E7	2	1	2
E8	3	1	1
E9	2	2	2
E10	2	2	2
E11	1	1	1
E12	3	3	4
E14	1	1	1
E15	2	2	1
E16	1	1	1
E17	2	1	1
E18	2	1	2
E19	2	2	3
E20	2	2	2
E21	1	2	2
E22	2	2	1

Table A3.10 (continued):

b) Day 3

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	24	26	24	42	53	51	ND	5903	5796
E2	21	8	19	30	24	23	8982	9428	8118
E3	28	52	25	118	105	141	ND	4659	3771
E5	42	40	37	150	117	93	6291	6800	6126
E6	36	42	38	83	107	93	5115	5340	5554
E7	67	79	91	337	235	414	9750	7569	8615
E8	53	52	52	134	123	112	6424	6919	6112
E9	80	79	79	789	687	493	4303	4074	3851
E10	147	163	107	646	531	550	12508	12504	12960
E11	77	108	99	345	276	311	13695	12675	12835
E12	37	73	42	306	253	248	13851	14484	13511
E14	94	68	72	294	262	182	14932	15683	15130
E15	54	52	49	335	349	307	14660	14043	13926
E16	184	172	135	417	426	388	13682	13332	14104
E17	61	114	79	122	110	179	12801	12310	12804
E18	76	98	66	456	423	318	7372	6266	5817
E19	76	108	90	307	316	323	10797	10305	10392
E20	66	72	82	417	333	323	7982	7610	7787
E21	81	59	62	171	169	157	6457	5732	6051
E22	104	135	113	257	253	170	10723	9941	10703

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	2	2	2
E2	1	3	1
E3	4	2	6
E5	4	3	3
E6	2	3	2
E7	5	3	5
E8	3	2	2
E9	10	9	6
E10	4	3	5
E11	4	3	3
E12	8	3	6
E14	3	4	3
E15	6	7	6
E16	2	2	3
E17	2	1	2
E18	6	4	5
E19	4	3	4
E20	6	5	4
E21	2	3	3
E22	2	2	2

c) Day 5

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	66	55	48	311	368	273	2712	2267	2276
E2	40	36	42	130	96	91	4000	3804	3333
E3	109	160	83	633	561	512	2146	1980	2178
E5	119	137	113	912	868	742	5279	4746	4027
E6	109	116	92	679	376	494	5184	4130	5366
E7	346	368	315	2892	2610	2218	4471	1883	3551
E8	49	71	45	469	533	530	4797	4574	6114
E9	269	308	190	5382	3970	2985	4421	4038	1813
E10	328	429	875	3397	2575	2870	2576	2340	2644
E11	226	250	285	1578	1289	1294	10180	10453	13021
E12	140	207	156	1756	1237	1604	13139	12077	11556
E14	110	111	100	1576	1483	1163	11121	12403	11578
E15	103	124	111	1850	1822	1315	10342	9660	11244
E16	272	417	456	1394	1667	1349	4100	3776	2812
E17	115	327	327	1368	1079	984	3911	4509	4135
E18	344	216	228	5873	4350	4213	8380	7585	4594
E19	239	362	262	2439	1774	2126	9150	8888	7802
E20	276	259	316	4284	3248	3621	5952	4546	5974
E21	ND	451	196	1332	1363	1275	5089	4168	4439
E22	235	265	174	1209	1264	1649	2280	2412	1796

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	5	7	6
E2	3	3	2
E3	6	4	6
E5	8	6	7
E6	6	3	5
E7	8	7	7
E8	10	8	12
E9	20	13	16
E10	10	6	3
E11	7	5	5
E12	13	6	10
E14	14	13	12
E15	18	15	12
E16	5	4	3
E17	12	3	3
E18	17	20	18
E19	10	5	8
E20	16	13	11
E21	ND	3	6
E22	5	5	9

Table A3.10 (continued):

d) Day 7

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	151	107	59	1373	850	1091	1010	1224	1387
E2	54	60	52	345	378	409	637	610	483
E3	336	385	328	1851	2005	1137	909	986	1425
E5	281	223	168	5127	3950	6066	714	635	615
E6	169	346	136	3273	2228	2839	942	887	726
E7	532	922	826	5770	5730	4831	966	937	948
E8	59	79	58	1296	1212	1371	1618	1751	1760
E9	245	384	245	9809	7225	8055	2112	1520	563
E10	310	459	974	6207	4606	6688	845	627	1226
E11	397	607	233	3335	2861	3354	2395	2651	3065
E12	309	351	221	5305	3993	5110	2769	2512	2699
E14	139	120	141	6677	6337	5054	1456	1681	1762
E15	202	360	244	5584	7079	4498	1169	1175	1129
E16	652	975	863	4317	4397	4419	1037	1182	949
E17	851	561	544	2622	2123	3792	1218	909	1226
E18	440	392	300	9115	7794	9592	2345	1763	2170
E19	ND	ND	ND	7654	7708	9046	1110	939	1745
E20	809	393	492	6552	6636	6721	1379	1032	1559
E21	290	ND	572	5609	5682	5723	1558	1366	1290
E22	374	531	387	3828	3996	4821	430	494	336

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	9	8	19
E2	6	6	8
E3	6	5	3
E5	18	18	36
E6	19	6	21
E7	11	6	6
E8	22	15	23
E9	40	19	33
E10	20	10	7
E11	8	5	14
E12	17	11	23
E14	48	53	36
E15	28	20	18
E16	7	5	5
E17	3	4	7
E18	21	20	32
E19	ND	ND	ND
E20	8	17	14
E21	19	ND	10
E22	10	8	12

e) Day 9

Donor	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
E1	315	243	154	1399	824	1274	851	1283	1536
E2	126	135	129	652	385	677	308	285	337
E3	341	366	370	1524	1490	728	691	510	1072
E5	973	429	626	6291	4314	8826	263	263	346
E6	679	379	161	4177	3010	1938	178	139	520
E7	1495	1201	1321	6780	5391	5121	381	270	350
E8	152	164	177	1683	1352	1711	1080	805	1298
E9	442	787	360	5368	6440	6666	600	647	276
E11	1660	1314	1183	2737	2524	2028	747	738	1381
E12	792	880	655	4298	3725	4264	447	456	1123
E14	251	586	292	6313	5704	5274	493	643	641
E15	480	870	695	6313	6319	6929	553	514	522
E18	1038	1029	877	5361	4836	5070	509	407	841
E19	263	247	81	4098	2889	3547	580	599	1029
E20	943	1391	1657	3152	2860	3738	577	275	524
E21	100	100	64	822	1315	2112	985	828	2080

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
E1	4	3	8
E2	5	3	5
E3	4	4	2
E5	6	10	14
E6	6	8	12
E7	5	4	4
E8	11	8	10
E9	12	8	19
E11	2	2	2
E12	5	4	7
E14	25	10	18
E15	13	7	10
E18	5	5	6
E19	16	12	44
E20	3	2	2
E21	8	13	33

Table A3.11: Complete lymphoproliferative results for PBMCs of immune individuals by day. Data is given in geometric mean cpm (left-hand tables) and SI (PfSE/uRBC with and without control IgG or αIL-12, right-hand tables)(ND = not done).

a) Day 3

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	13	22	12	16	36	40	26	4609	23
I2	46	36	44	26	186	76	86	11576	266
I3	10	12	7	21	52	53	48	8447	292
I4	20	24	10	8	60	53	78	7031	43
I5	33	31	49	47	95	78	62	13871	803
I6	37	37	66	43	73	62	48	14961	110
I7	239	216	185	140	345	70	14	557	119
I8	38	32	13	17	71	25	243	24152	175
I9	17	58	44	12	91	40	95	34928	816
I10	79	55	130	57	112	117	128	33922	863
I11	9	20	11	12	49	48	27	8528	36
I12	21	22	50	35	53	64	44	3842	811
I13	93	71	61	104	214	143	81	8618	896
I14	81	72	97	100	274	203	228	37243	359
I15	35	37	43	46	165	142	140	29109	386
I16	83	80	84	66	80	85	58	3986	139
I17	20	12	27	91	335	35	230	12624	2601
I18	21	17	27	13	85	66	36	33959	690

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	2	3	2
I2	5	2	3
I3	4	7	2
I4	2	6	9
I5	3	2	1
I6	2	1	1
I7	2	0	0
I8	2	2	15
I9	2	1	8
I10	2	1	2
I11	2	4	2
I12	2	1	1
I13	3	2	1
I14	4	2	2
I15	4	3	3
I16	1	1	1
I17	27	1	3
I18	5	2	3

b) Day 5

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	9	10	8	25	33	ND	ND	320	4822
I2	32	59	381	194	670	11	23	2849	6800
I3	16	48	74	44	368	297	1102	15839	2571
I4	27	26	53	122	703	675	1227	6003	140
I5	60	65	161	158	2081	1693	1723	41494	7382
I7	56	67	308	241	1150	1639	1223	15133	5447
I8	1	ND	15	52	338	609	268	39717	2241
I11	9	27	33	34	155	199	102	25756	306
I13	322	214	612	634	2738	1427	728	8000	3360
I14	138	328	403	542	1087	199	976	46856	1342
I15	25	36	68	67	1046	971	413	34082	1758
I16	36	70	49	58	53	14	32	2723	50
I18	94	149	265	143	557	340	202	33125	1225

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	3	ND	ND
I2	11	0	0
I3	8	4	25
I4	28	13	10
I5	32	11	11
I7	17	5	5
I8	ND	40	5
I11	6	6	3
I13	13	2	1
I14	3	0	2
I15	29	14	6
I16	1	0	1
I18	4	1	1

Table A3.11 (continued):

c) Day 7

Donor	CM	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PPD
I1	6	16	14	32	92	101	51	2031	29756
I2	123	117	803	1754	11763	14414	8908	13733	21059
I3	75	146	255	213	1413	1502	458	20	10255
I4	32	60	373	453	7186	5590	559	31	483
I5	197	90	321	166	4896	4332	1267	18651	35763
I6	100	99	311	102	294	417	153	13533	1067
I7	168	206	2768	979	7319	5922	5231	13819	18142
I8	129	188	770	181	1977	1486	940	9968	13377
I9	652	481	895	501	1153	967	394	11974	12153
I10	38	100	331	254	2493	2352	1030	15755	26201
I11	65	92	298	82	439	445	294	15420	1589
I12	75	143	67	190	547	778	480	12738	41973
I13	135	1151	2894	1888	7712	5832	4360	5150	19229
I14	211	442	468	309	7816	6185	8162	9893	6918
I15	13	47	56	132	6358	4328	895	17436	17943
I16	26	42	23	45	51	82	34	3251	74
I17	31	61	730	677	14005	6291	5511	11138	36573
I18	156	99	472	288	1584	822	436	2557	7376
I19	66	79	654	876	17095	13220	8276	7379	21411
I20	21	23	323	694	952	894	475	3316	4713

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
I1	6	7	2
I2	100	18	5
I3	10	6	2
I4	120	15	1
I5	54	14	8
I6	3	1	1
I7	36	2	5
I8	11	2	5
I9	2	1	1
I10	25	7	4
I11	5	1	4
I12	4	12	3
I13	7	2	2
I14	18	13	26
I15	134	78	7
I16	1	4	1
I17	229	9	8
I18	16	2	2
I19	217	20	9
I20	42	3	1

Table A3.12: Complete lymphoproliferative results for PBMCs of naïve individuals used as controls in Ghana by day. Data is given in geometric mean cpm (left-hand tables) and SI (PfSE/uRBC with and without control IgG or αIL-12, right-hand tables)(ND = not done).

a) Day 2

DAY	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	404	403	415	438	408	381	2873	5435	5068
C4	24	10	30	58	56	50	4499	6539	7966

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	1	1	1
C4	2	5	2

b) Day 3

DAY	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	160	157	122	164	167	164	3967	8331	7573
C3	28	25	24	60	75	54	3882	5485	5628
C4	32	38	32	114	149	139	8257	12579	12995
C5	104	25	77	397	214	267	13252	ND	ND

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	1	1	1
C3	2	3	2
C4	4	4	4
C5	4	9	3

c) Day 5

DAY	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	73	60	45	196	120	199	2825	6683	3307
C3	42	39	35	361	306	333	2741	4871	5792
C4	135	90	76	935	633	646	7393	7541	8563

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	3	2	4
C3	9	8	9
C4	7	7	8

d) Day 7

DAY	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	91	86	53	239	502	284	1142	971	1650
C3	65	54	50	986	670	855	1570	1724	1689
C4	80	142	80	1310	894	908	3012	1671	1860
C5	38	175	49	5561	3545	3484	3203	ND	ND

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	3	6	5
C3	15	12	17
C4	16	6	11
C5	146	20	71

e) Day 9

DAY	RBC	RBC+ IgG	RBC+ αIL-12	PfSE	PfSE+ IgG	PfSE+ αIL-12	PHA	PHA+ IgG	PHA+ αIL-12
C2	207	163	114	1436	1385	733	863	437	598
C3	98	61	69	2186	1513	1777	487	1020	1242
C4	187	424	386	1753	1776	2197	1265	1184	2235

Donor	PfSE	PfSE+ IgG	PfSE+ αIL-12
C2	7	8	6
C3	22	25	26
C4	9	4	6

Table A3.13: Complete IL-10 (pg/ml) results for PBMCs of naïve individuals by day. Data is given in arithmetic mean (left-hand tables) and Ag-specific responses (Ag-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	PfSE	PHA
N2	48	45	76
N3	42	42	44
N4	55	50	61
N5	41	42	45
N12	54	53	ND
N13	108	145	ND
N14	45	50	ND
N15	44	48	ND
N16	46	62	ND
N17	40	46	ND
N18	48	64	ND
N19	50	66	ND

Donor	PfSE	PHA
N2	-3	28
N3	0	2
N4	-5	6
N5	1	4
N12	-1	ND
N13	36	ND
N14	6	ND
N15	4	ND
N16	16	ND
N17	6	ND
N18	16	ND
N19	17	ND

b) Day 2

Donor	RBC	PfSE	PHA
N2	46	45	371
N3	73	86	233
N4	86	69	297
N5	46	54	142
N6	100	79	263
N7	103	111	ND
N8	51	55	72
N9	233	209	540
N10	46	44	91
N11	46	44	102
N12	44	48	127
N13	86	100	160
N14	51	49	112
N15	ND	49	63
N16	44	49	158
N17	41	42	145
N18	42	49	136
N19	50	50	190

Donor	PfSE	PHA
N2	-1	325
N3	12	160
N4	-17	211
N5	8	97
N6	-20	163
N7	7	ND
N8	4	21
N9	-24	306
N10	-2	45
N11	-3	56
N12	4	83
N13	14	74
N14	-2	61
N15	ND	ND
N16	5	114
N17	0	104
N18	7	95
N19	-1	140

Table A3.13 (continued):

c) Day 4

Donor	RBC	PfSE	PHA
N2	43	50	284
N3	85	68	229
N4	108	75	577
N5	50	63	145
N6	88	70	510
N7	106	93	302
N8	50	58	127
N9	218	174	486
N10	43	49	179
N11	47	43	111
N12	47	53	178
N13	65	90	152
N14	52	50	108
N15	44	50	70
N16	44	49	120
N17	42	41	107
N18	43	51	147
N19	42	52	164

Donor	PfSE	PHA
N2	7	241
N3	-17	144
N4	-33	470
N5	13	96
N6	-18	423
N7	-13	196
N8	8	77
N9	-44	267
N10	6	136
N11	-3	64
N12	6	131
N13	24	87
N14	-3	56
N15	5	25
N16	5	76
N17	-1	64
N18	8	104
N19	9	122

d) Day 6

Donor	RBC	PfSE	PHA
N2	46	67	193
N3	92	77	188
N4	113	77	321
N5	41	57	111
N6	88	69	346
N7	74	100	202
N8	52	45	109
N9	125	155	226
N10	46	48	123
N11	47	43	108
N12	46	49	130
N13	69	91	103
N14	52	48	97
N15	46	47	68
N16	ND	47	107
N17	41	41	83
N18	44	44	104
N19	43	48	153

Donor	PfSE	PHA
N2	22	147
N3	-15	96
N4	-36	208
N5	16	70
N6	-19	257
N7	26	128
N8	-7	57
N9	30	101
N10	2	77
N11	-4	61
N12	4	84
N13	22	34
N14	-4	45
N15	0	22
N16	ND	ND
N17	0	42
N18	0	60
N19	5	110

Table A3.13 (continued):

e) Day 8

Donor	RBC	PfSE	PHA
N2	48	75	91
N3	80	103	110
N4	111	69	218
N5	43	54	89
N6	78	72	190
N7	72	110	262
N8	49	46	116
N9	94	107	107
N10	45	47	122
N11	47	44	78
N12	45	45	107
N13	64	74	95
N14	54	51	99
N15	47	45	63
N16	44	48	96
N17	43	43	89
N18	44	43	110
N19	44	48	147

Donor	PfSE	PHA
N2	27	43
N3	23	30
N4	-43	107
N5	12	46
N6	-7	112
N7	39	190
N8	-3	67
N9	13	13
N10	1	76
N11	-2	32
N12	0	62
N13	10	32
N14	-3	46
N15	-3	15
N16	4	52
N17	0	46
N18	0	66
N19	4	104

Table A3.14: Complete IL-10 (pg/ml) results for PBMCs of exposed individuals by day. Data is given in arithmetic mean (left-hand tables and Ag-specific responses (Ag-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	PfSE	PHA
E1	42	45	ND
E2	56	68	85
E3	51	55	ND
E5	108	106	163
E6	150	132	85
E7	65	71	85
E8	48	48	46
E9	44	44	54
E10	72	70	138
E11	53	57	87
E12	64	58	95
E14	68	62	83
E15	139	130	247
E16	53	56	112
E17	112	75	191
E18	44	48	55
E19	115	113	102
E20	64	62	75
E21	50	53	56
E22	66	59	98

Donor	PfSE	PHA
E1	3	ND
E2	12	29
E3	3	ND
E5	-2	55
E6	-19	-66
E7	6	19
E8	0	-2
E9	0	10
E10	-2	66
E11	4	34
E12	-6	31
E14	-6	15
E15	-9	108
E16	3	58
E17	-36	79
E18	4	11
E19	-1	-13
E20	-2	11
E21	2	6
E22	-7	32

Table A3.14 (continued):

b) Day 2

Donor	RBC	PfSE	PHA
E1	46	42	ND
E2	58	64	134
E3	53	54	ND
E5	68	66	161
E6	65	64	101
E7	75	88	130
E8	44	47	52
E9	48	53	73
E10	78	90	256
E11	53	63	304
E12	62	76	247
E14	70	74	304
E15	143	209	505
E16	57	69	549
E17	209	76	207
E18	50	50	98
E19	ND	70	125
E20	69	76	120
E21	45	45	65
E22	73	66	196

Donor	PfSE	PHA
E1	-4	ND
E2	6	76
E3	1	ND
E5	-3	93
E6	-1	37
E7	13	54
E8	3	8
E9	5	25
E10	11	178
E11	10	251
E12	14	185
E14	4	234
E15	66	362
E16	11	491
E17	-132	-2
E18	0	48
E19	ND	ND
E20	8	52
E21	1	21
E22	-7	123

c) Day 4

Donor	RBC	PfSE	PHA
E1	44	43	64
E2	59	69	138
E3	51	54	66
E5	72	61	158
E6	71	68	80
E7	69	86	150
E8	42	47	60
E9	47	50	80
E10	63	85	302
E11	47	49	438
E12	58	60	320
E14	48	56	255
E15	92	96	400
E16	67	75	1373
E17	182	ND	120
E18	52	51	129
E19	ND	63	124
E20	58	74	113
E21	ND	47	77
E22	73	72	407

Donor	PfSE	PHA
E1	0	20
E2	10	79
E3	3	15
E5	-10	87
E6	-3	9
E7	17	81
E8	4	18
E9	3	33
E10	22	239
E11	2	390
E12	2	263
E14	8	207
E15	4	308
E16	8	1306
E17	ND	-62
E18	-1	77
E19	ND	ND
E20	16	55
E21	ND	ND
E22	-1	334

Table A3.14 (continued):

d) Day 6

Donor	RBC	PfSE	PHA
E1	44	45	51
E2	55	54	123
E3	50	52	70
E5	53	52	81
E6	64	80	114
E7	ND	ND	156
E8	44	47	65
E9	43	48	87
E10	60	73	174
E11	47	48	242
E12	55	68	265
E14	49	60	304
E15	69	91	439
E16	63	77	988
E17	115	53	107
E18	55	48	120
E19	ND	59	124
E20	ND	66	121
E21	44	43	69
E22	69	67	287

Donor	PfSE	PHA
E1	1	8
E2	0	68
E3	2	20
E5	-1	28
E6	16	50
E7	ND	ND
E8	2	21
E9	6	44
E10	13	114
E11	1	194
E12	13	211
E14	11	255
E15	22	370
E16	15	926
E17	-62	-8
E18	-8	65
E19	ND	ND
E20	ND	ND
E21	-1	25
E22	-2	218

e) Day 8

Donor	RBC	PfSE	PHA
E1	45	43	56
E2	51	47	138
E3	ND	ND	71
E5	54	53	74
E6	53	66	87
E7	ND	ND	153
E8	43	42	61
E9	42	49	72
E10	57	65	152
E11	47	55	207
E12	48	60	173
E14	47	76	229
E15	64	105	384
E16	ND	ND	698
E17	99	150	79
E18	46	56	76
E19	ND	73	126
E20	59	71	184
E21	44	78	80
E22	68	60	323

Donor	PfSE	PHA
E1	-2	11
E2	-4	87
E3	ND	ND
E5	-1	20
E6	12	34
E7	ND	ND
E8	-1	18
E9	7	29
E10	7	95
E11	8	160
E12	12	125
E14	29	181
E15	41	320
E16	ND	ND
E17	52	-19
E18	10	30
E19	ND	ND
E20	12	125
E21	34	36
E22	-9	255

Table A3.15: Complete IL-10 (pg/ml) results for PBMCs of immune individuals by day. Data is given in arithmetic mean (left-hand tables) and Ag-specific responses (Ag-uRBC, right-hand tables)(ND = not done).

a) Day 2

Donor	RBC	PfSE	PHA
I1	39	39	45
I2	40	42	52
I3	39	40	57
I4	41	42	50
I5	46	51	66
I6	41	42	51
I7	43	43	64
I8	43	42	60
I9	46	51	160
I10	43	47	86
I11	40	42	65
I12	40	41	47
I13	40	43	53
I14	43	44	124
I15	41	41	71
I16	40	41	41
I17	40	41	66
I18	42	45	108

Donor	PfSE	PHA
I1	0	6
I2	2	11
I3	1	18
I4	1	9
I5	5	19
I6	2	10
I7	0	21
I8	-1	17
I9	5	114
I10	4	43
I11	2	25
I12	1	7
I13	3	13
I14	2	81
I15	0	30
I16	1	1
I17	1	26
I18	3	66

b) Day 4

Donor	RBC	PfSE	PHA
I1	39	39	42
I2	41	43	51
I3	39	40	52
I4	42	43	46
I5	43	48	72
I7	42	44	66
I8	41	42	74
I11	41	43	81
I13	40	43	45
I14	41	43	91
I15	41	42	52
I16	40	42	40
I18	42	43	64

Donor	PfSE	PHA
I1	0	3
I2	1	10
I3	1	13
I4	1	4
I5	5	29
I7	2	24
I8	1	33
I11	2	40
I13	3	5
I14	2	50
I15	1	11
I16	2	0
I18	1	22

Table A3.15 (continued):

c) Day 6

Donor	RBC	PfSE	PHA
I1	39	39	42
I2	41	46	51
I3	40	41	53
I4	41	44	46
I5	45	49	68
I6	40	42	52
I7	41	43	60
I8	40	42	63
I9	43	47	95
I10	43	45	108
I11	40	45	79
I12	40	42	49
I13	41	42	46
I14	42	43	74
I15	40	41	52
I16	41	43	40
I17	41	44	57
I18	42	44	81
I19	40	43	168
I20	41	43	99

Donor	PfSE	PHA
I1	1	3
I2	5	10
I3	0	12
I4	4	5
I5	4	23
I6	2	12
I7	2	18
I8	2	23
I9	4	51
I10	2	65
I11	4	39
I12	2	9
I13	2	5
I14	2	32
I15	1	12
I16	2	-1
I17	3	16
I18	2	39
I19	3	128
I20	2	58

Table A3.16: Complete IL-10 (pg/ml) results for PBMCs of naïve individuals used as controls in Ghana by day. Data is given in arithmetic mean (left-hand tables) and Ag-specific responses (Ag-uRBC, right-hand tables)(ND = not done).

a) Day 1

Donor	RBC	PfSE	PHA
C2	86	78	125
C4	49	50	60

Donor	PfSE	PHA
C2	-9	39
C4	1	11

b) Day 2

Donor	RBC	PfSE	PHA
C2	81	103	146
C3	56	66	72
C4	49	54	97
C5	46	54	96

Donor	PfSE	PHA
C2	22	65
C3	9	15
C4	5	48
C5	8	50

c) Day 4

Donor	RBC	PfSE	PHA
C2	84	127	112
C3	74	87	94
C4	55	62	92

Donor	PfSE	PHA
C2	43	28
C3	13	20
C4	7	38

d) Day 6

Donor	RBC	PfSE	PHA
C2	80	178	108
C3	65	72	67
C4	57	61	73
C5	47	51	64

Donor	PfSE	PHA
C2	98	27
C3	6	2
C4	4	17
C5	4	18

e) Day 8

Donor	RBC	PfSE	PHA
C2	81	136	127
C3	70	61	88
C4	53	58	62

Donor	PfSE	PHA
C2	55	46
C3	-10	18
C4	5	8

Appendix 4

Table A4.1: Total number of cells cultured and harvested for each naïve donor for phenotypic analysis (cells are indicated as 10^5).

	Day0	Day3						Day7							
		RBC		PfSE		CM		RBC		PfSE		PHA		PPD	
Donor	Total	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested
N3	50	20	10.5	20	10.3	20	11.0	20	6.9	20	9.0	20	12.4	20	8.8
N7	50	20	13.8	20	15.0	20	10.2	20	10.5	20	13.8	20	13.8	20	5.2
N8	50	20	10.3	20	12.3	20	11.3	20	8.3	20	17.3	20	14.0	20	14.0
N13	50	20	8.8	20	11.0	20	4.7	20	5.2	20	10.2	20	6.9	20	4.7
N14	50	20	14.0	20	19.8	20	8.8	20	16.0	20	17.1	20	12.4	20	12.1
N16	50	20	14.0	20	13.8	20	8.0	20	11.6	20	14.6	20	8.0	20	9.6
N17	50	20	14.0	20	13.8	20	7.7	20	4.4	20	9.9	20	12.7	20	10.2
N18	50	20	9.3	20	10.5	20	4.1	20	3.6	20	5.0	20	15.4	20	6.9
N19	50	20	8.5	20	13.3	20	11.6	20	25.6	20	15.7	20	25.9	20	10.2
N20	50	20	16.8	20	14.5	20	4.4	20	10.2	20	7.2	20	30.8	20	14.0

Table A4.2: Total number of cells cultured and harvested for each exposed donor for phenotypic analysis (cells are indicated as 10^5).

	Day 0	Day 7									
		CM		RBC		PfSE		PHA		PPD	
Donor	Total	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested
E1	20	40	9.0	40	15.0	40	12.2	40	17.0	40	11.8
E2	20	40	8.8	40	8.4	40	8.0	40	14.8	40	8.8
E3	20	40	18.6	40	19.0	40	17.6	40	17.4	40	24.0
E4	20	40	15.2	40	15.0	40	18.6	40	11.2	40	19.0
E5	20	40	11.6	40	21.4	40	11.2	40	29.0	40	18.2
E6	20	40	19.6	40	16.8	40	16.2	40	19.4	40	19.4
E7	20	40	12.0	40	13.8	40	13.6	40	14.4	40	9.0
E8	20	40	14.2	40	8.0	40	13.0	40	12.8	40	19.0
E9	20	40	22.4	40	30.2	40	17.6	40	23.0	40	19.6
E10	20	40	10.4	40	7.2	40	12.0	40	8.0	40	13.4
E11	20	40	12.8	40	11.8	40	12.0	40	8.0	40	14.2
E12	20	40	10.0	40	7.4	40	13.6	40	8.6	40	20.6
E14	20	40	14.2	40	17.4	40	15.0	40	15.2	40	8.2
E15	20	40	19.4	40	8.8	40	11.2	40	13.4	40	23.4
E16	20	40	24.2	40	28.2	40	18.0	40	44.4	40	24.8
E17	20	40	17.2	40	13.0	40	14.6	40	22.0	40	15.2
E18	20	40	14.8	40	16.2	40	17.0	40	11.0	40	10.4
E19	20	40	11.6	40	11.6	40	13.8	40	30.2	40	22.0
E20	20	40	14.0	40	22.0	40	15.2	40	20.8	40	51.0
E21	20	40	15.8	40	16.2	40	22.0	40	22.4	40	20.4

Table A4.3: Total number of cells cultured and harvested for each immune donor for phenotypic analysis (cells are indicated as 10^5).

	Day 0	Day 3				Day 7			
		RBC		PfSE		RBC		PfSE	
Donor	Total	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested
I1	4	10	3.2	10	1.7	10	3.8	10	1.7
I2	4	5	2.4	5	1.8	5	1.7	5	2.0
I3	4	25	14.7	25	1.7	25	2.7	25	4.5
I4	4	10	4.2	10	2.3	10	1.1	10	1.1
I5	4	50	24.2	50	20.0	50	15.6	50	9.8
I7	4	10	3.3	10	3.5	10	3.8	10	2.7
I8	4	5	3.8	5	2.3	5	0.8	5	1.8
I9	4	5	1.1	5	3.0	5	1.4	5	1.7
I11	4	5	3.0	5	1.2	5	2.3	5	1.4
I12	4	5	0.9	5	1.1	5	2.1	5	4.1
I13	4	25	3.2	25	4.1	25	5.4	25	6.3
I14	4	10	2.7	10	1.2	10	2.3	10	2.3
I15	4	25	4.7	25	6.5	25	2.3	25	2.0
I16	4	5	2.0	5	0.6	5	1.2	5	0.6
I17	4	5	0.3	5	0.6	5	1.2	5	1.2
I18	4	10	1.4	10	1.1	10	1.5	10	0.9
I19	4	5	ND	5	ND	5	1.7	5	3.2
I20	4	5	ND	5	ND	5	1.2	5	2.3

Table A4.4: Total number of cells cultured and harvested for each naïve donor used as controls in Ghana for phenotypic analysis (cells are indicated as 10^5).

	Day 0	Day 7									
		CM		RBC		PfSE		PHA		PPD	
Donor	Total	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested	Cultured	Harvested
C1	20	20	10	20	8.4	20	8	20	21.6	20	6
C2	20	40	5.3	40	7.6	40	8.5	40	15	40	5.5
C4	20	20	3.2	40	5.1	40	11	40	11.2	40	12.7

Table A4.5: Summary data for percentages of cellular subsets stained on a) day 0, b) day 3 and c) day 7 for naïve individuals (arithmetic mean and SEM indicated). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
% in gate	83.59	8.33
	1.17	0.62
CD20	5.23	2.50
	0.56	0.43
CD56	1.73	7.40
	0.29	1.52
CD56/CD3	0.37	4.71
	0.08	1.06
Total CD3	77.97	64.36
	2.44	2.88
CD45RA/CD3	51.26	31.99
	3.69	2.11
CD45RO/CD3	55.12	62.70
	2.56	2.65
TcRαβ/CD3	67.07	21.81
	2.45	2.24
TcRγδ/CD3	3.81	2.53
	0.84	0.48
CD4/CD3	43.50	56.69
	3.67	2.61
CD8/CD3	19.90	28.35
	2.15	2.32

Table A4.5 (continued):

b)

	RBC		PFSE	
	R1	R2	R1	R2
% in gate	86.80	1.49	82.61	1.91
	1.16	0.09	1.34	0.15
CD20	1.02	2.73	0.85	3.56
	0.23	0.63	0.18	0.78
CD56	0.22	0.20	0.47	2.20
	0.06	0.16	0.11	0.57
CD56/CD3	0.08	0.08	0.14	0.24
	0.06	0.08	0.10	0.17
CD3	81.82	81.54	80.61	72.34
	2.01	2.28	1.97	2.45

Table A4.5 (continued):

c)

	CM		RBC		PfSE		PHA		PPD	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
% in gate	39.04	0.52	39.33	0.45	43.60	7.95	10.98	16.12	30.44	10.12
	2.42	0.11	2.65	0.09	2.49	1.02	1.20	0.78	4.30	2.71
CD20	5.50	13.78	5.71	14.63	10.72	7.66	3.02	2.94	6.72	6.16
	1.11	4.42	1.00	3.17	2.10	1.61	0.60	0.77	1.45	1.50
CD56	1.98	2.51	1.84	3.68	3.33	2.51	4.47	0.19	4.94	2.04
	0.65	1.15	0.67	1.72	0.75	0.74	0.49	0.05	0.70	0.51
CD56/CD3	1.39	0.94	1.34	2.19	0.63	0.81	0.45	0.10	1.53	0.26
	0.64	0.54	0.67	1.56	0.22	0.52	0.07	0.03	0.83	0.09
Total CD3	83.78	55.29	83.92	62.80	79.11	83.44	82.26	93.76	84.03	83.38
	2.22	7.17	1.83	5.09	2.60	2.77	2.11	1.57	1.43	3.85
CD45RA/CD3	42.81	27.00	42.19	35.13	41.83	16.51	31.23	34.14	46.52	20.72
	2.69	3.54	3.95	7.13	3.16	1.05	2.91	2.49	3.22	2.70
CD45RO/CD3	17.81	25.54	16.78	18.60	17.13	71.98	31.03	42.78	21.38	67.95
	3.52	6.59	3.18	6.45	2.82	3.52	3.53	5.30	2.97	7.08
TcRαβ/CD3	25.56	27.62	25.14	25.97	26.33	52.82	27.12	56.84	30.92	56.25
	3.86	2.95	4.03	5.11	4.50	4.65	3.94	4.61	4.17	6.00
TcRγδ/CD3	4.92	5.75	4.73	2.17	3.85	11.53	4.21	8.07	4.97	11.24
	0.90	2.45	1.26	1.13	0.67	0.77	0.63	1.13	0.99	2.03
CD4/CD3	51.10	34.98	50.97	31.80	47.58	79.39	44.19	61.62	53.06	76.64
	3.25	4.01	3.45	4.32	3.22	2.61	3.85	4.54	2.31	5.63
CD8/CD3	15.20	8.98	14.49	8.55	16.08	2.24	24.02	30.32	17.72	6.55
	2.98	2.42	2.94	2.00	3.33	0.56	4.07	4.07	3.31	2.18

Table A4.6: Summary data for total numbers of cells of cellular subsets stained on a) day 0, b) day 3 and c) day 7 for naïve individuals (arithmetic mean and SEM indicated, cells are per 10^6 cells plated on day 0). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
cells in gate	835890	83290
	11671	6247
CD20	43691	2016
	4786	280
CD56	14464	5901
	2362	1162
CD56/CD3	3083	3770
	648	819
Total CD3	653704	53726
	27286	4898
CD45RA/CD3	431477	26490
	35693	2374
CD45RO/CD3	460364	52386
	21344	4767
TcRab/CD3	562723	17812
	26590	1898
TcRgd/CD3	31648	2085
	6932	373
CD4/CD3	367044	46881
	35011	3586
CD8/CD3	165440	23755
	17349	2679

Table A4.6 (continued):

b)

	RBC		PFSE	
	R1	**R2**	**R1**	**R2**
cells in gate	501282	8413	534840	12461
	44938	726	43645	1054
CD20	4737	225	4351	425
	1066	48	1311	94
CD56	982	18	2501	274
	201	15	517	68
CD56/CD3	310	7	703	32
	219	7	490	23
CD3	413835	6909	429122	9031
	41866	720	39260	963

Table A4.6 (continued):

c)

	CM		RBC		PfSE		PHA		PPD	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
cells in gate	162397	2227	208069	2554	269361	48125	80117	124188	136778	53519
	23119	642	52689	886	39035	8689	12840	21326	19391	17437
CD20	9351	362	13739	312	31013	3163	2396	3585	11150	1714
	2687	133	5792	113	9218	658	537	1104	4520	370
CD56	2526	109	3118	79	8496	796	3358	243	6129	1061
	444	58	1063	32	2199	138	469	93	832	337
CD56/CD3	1513	30	1875	19	1725	145	366	118	1880	176
	455	18	838	10	752	79	111	38	993	73
Total CD3	137251	1367	175481	1681	213098	41721	66439	116333	114512	48909
	20904	554	44109	615	30834	8324	11429	19479	15315	16965
CD45RA/CD3	71254	607	101966	1086	118620	8042	24990	43489	67215	8869
	11188	261	33553	575	22530	1434	4681	8339	14276	2669
CD45RO/CD3	29989	642	26291	472	42846	36706	23651	49971	26590	43974
	9421	255	5314	151	9135	7982	4406	8643	3766	15806
TcRαβ/CD3	44724	699	52743	846	74040	28065	20224	68290	40365	35890
	9112	224	12530	353	15697	6740	3376	9059	5269	12219
TcRγδ/CD3	6964	94	8017	36	10350	6017	3363	10216	6493	7250
	1135	43	2196	16	2075	1256	684	2579	1315	2782
CD4/CD3	87245	873	114422	1037	135161	39499	36368	76081	74129	47282
	15725	374	32010	477	24444	7850	7282	11938	11496	16865
CD8/CD3	22945	210	25410	224	35915	1000	18500	38603	22632	1323
	4908.74	98.95	5343.13	62.31	5167.48	243.46	3463.00	9433.94	3976.45	314.05

Table A4.7: Summary data for percentages of cellular subsets stained on a) day 0 and b) day 7 for exposed individuals (arithmetic mean and SEM indicated). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
% in gate	44.69	7.78
	3.91	2.08
CD20	10.05	16.71
	1.23	2.64
CD20/CD45RO	0.07	0.46
	0.04	0.10
CD56	0.86	3.15
	0.44	1.01
CD56/CD45RO	0.44	1.11
	0.41	0.78
Total CD3	58.21	52.52
	2.47	2.93
CD45RA/CD3	31.44	24.13
	1.92	2.08
CD45RO/CD3	20.49	30.90
	1.78	2.25
TcRαβ/CD3	1.49	3.18
	0.42	0.70
TcRαβ/CD45RO	0.20	1.19
	0.05	0.20
TcRγδ/CD3	3.22	5.64
	0.62	0.70
TcRγδ/CD45RO	0.35	2.33
	0.05	0.38
CD4/CD3	31.60	36.54
	2.67	2.70
CD4/CD45RO	10.66	19.48
	1.32	2.15
CD8/CD3	15.61	28.93
	1.68	1.69
CD8/CD45RO	1.92	4.79
	0.84	1.14
TcR Vγ9/CD3	3.62	4.68
	1.03	1.43
TcR Vγ9/CD45RO	0.26	1.25
	0.10	0.28
TcR Vδ1/CD3	2.77	3.95
	0.62	0.74
TcR Vδ1/CD45RO	0.04	0.79
	0.01	0.20

Table A4.7 (continued):

b)

	CM		RBC		PfSE		PHA		PPD	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
% in gate	39.01	4.14	40.16	3.27	39.04	4.64	29.79	16.74	36.96	7.38
	1.70	0.92	2.10	0.42	1.74	0.47	1.19	1.76	1.67	1.09
CD20	7.03	11.62	6.23	10.13	6.57	13.67	8.95	23.40	5.82	13.48
	1.23	1.69	0.92	1.61	0.83	1.94	1.46	3.34	0.83	1.63
CD20/CD45RO	0.07	0.84	0.04	1.24	0.05	1.25	0.55	3.37	0.05	1.39
	0.01	0.16	0.01	0.29	0.01	0.22	0.44	1.72	0.02	0.25
CD56	3.60	8.68	3.56	8.34	4.25	12.18	5.20	7.96	5.27	13.18
	0.88	1.52	0.97	1.57	0.89	1.57	2.15	3.64	1.15	1.47
CD56/CD45RO	0.19	1.30	0.15	1.73	0.14	2.38	1.11	3.02	0.18	2.69
	0.05	0.38	0.04	0.55	0.04	0.71	0.97	2.02	0.05	0.59
Total CD3	45.46	54.88	44.60	53.41	44.30	57.52	45.14	69.70	46.40	63.72
	4.00	5.05	4.11	4.22	3.91	4.13	4.74	3.69	3.94	3.82
CD45RA/CD3	21.61	29.43	21.11	28.19	19.61	19.51	14.46	27.35	19.95	16.49
	2.39	3.23	2.46	2.69	2.31	2.42	2.14	2.74	2.15	2.19
CD45RO/CD3	15.45	33.38	15.67	31.55	16.51	42.86	21.72	43.86	19.01	51.65
	1.70	3.78	1.76	3.26	1.70	3.43	2.33	2.55	2.05	3.86
TcRαβ/CD3	0.65	5.74	0.59	4.86	0.71	8.26	4.12	16.51	1.85	10.38
	0.23	1.20	0.21	1.37	0.25	1.54	1.59	3.07	0.87	2.20
TcRαβ/CD45RO	0.28	4.80	0.24	2.81	0.35	7.43	1.44	9.77	0.90	9.29
	0.09	0.78	0.09	0.57	0.12	1.36	0.45	1.32	0.40	1.81
TcRγδ/CD3	2.38	4.52	2.23	5.75	2.04	5.74	2.31	4.10	2.52	6.37
	0.46	0.65	0.49	1.15	0.51	0.77	0.63	1.15	0.54	1.17
TcRγδ/CD45RO	0.45	3.15	0.34	4.23	0.45	4.32	0.87	2.96	0.61	4.92
	0.10	0.39	0.06	1.08	0.09	0.61	0.37	1.13	0.17	1.07
CD4/CD3	29.52	40.44	30.05	40.33	28.11	43.60	21.20	36.86	31.09	52.60
	2.49	3.48	2.80	3.46	2.92	3.49	2.35	2.99	2.80	3.53
CD4/CD45RO	13.12	26.83	13.70	27.68	13.16	35.18	11.56	24.23	16.11	45.02
	1.56	2.87	1.71	3.04	1.40	3.16	1.51	2.40	1.93	3.75
CD8/CD3	12.56	28.23	12.12	26.11	13.34	25.63	20.45	42.32	12.34	16.90
	1.53	3.21	1.59	2.93	1.52	3.08	3.45	4.52	1.39	1.57
CD8/CD45RO	1.46	10.28	1.34	9.60	1.72	11.61	3.75	11.11	1.66	9.44
	0.26	1.77	0.25	1.37	0.30	1.41	1.01	1.53	0.44	1.00
TcR Vγ9/CD3	1.26	3.78	1.28	3.20	1.38	3.54	3.38	5.35	1.43	3.28
	0.21	0.42	0.21	0.39	0.32	0.82	2.11	3.09	0.29	0.86
TcR Vγ9/CD45RO	0.27	2.20	0.26	1.79	0.36	2.46	0.96	2.29	0.34	2.56
	0.05	0.35	0.06	0.29	0.09	0.72	0.41	0.91	0.16	0.88
TcR Vδ1/CD3	1.46	2.29	1.46	2.25	1.55	3.62	1.90	3.78	1.91	4.90
	0.34	0.48	0.35	0.45	0.39	0.90	0.55	1.29	0.47	1.30
TcR Vδ1/CD45RO	0.14	1.25	0.11	1.04	0.15	2.38	0.54	2.56	0.37	3.35
	0.07	0.31	0.04	0.24	0.06	0.92	0.38	1.46	0.21	1.20

Table A4.8: Summary data for total numbers of cells of cellular subsets stained on a) day 0 and b) day 7 for exposed individuals (arithmetic mean and SEM indicated, cells are per 10^6 cells plated on day 0). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
cells in gate	446906	77839
	39128	20839
CD20	45036	13135
	6505	4208
CD20/CD45RO	280	264
	128	68
CD56	3483	2128
	1542	647
CD56/CD45RO	1544	549
	1386	361
Total CD3	263549	45614
	27631	15876
CD45RA/CD3	142431	21700
	16436	8308
CD45RO/CD3	90857	26431
	11629	8509
TcRαβ/CD3	6174	2994
	2003	1371
TcRαβ/CD45RO	823	958
	265	321
TcRγδ/CD3	15954	5202
	3886	1671
TcRγδ/CD45RO	1531	1935
	265	505
CD4/CD3	138164	29161
	17507	9384
CD4/CD45RO	45853	15307
	6867	4949
CD8/CD3	71849	23729
	11433	6791
CD8/CD45RO	9817	3732
	4803	1125
TcR Vγ9/CD3	17619	4189
	5697	1643
TcR Vγ9/CD45RO	1092	1087
	388	383
TcR Vδ1/CD3	13288	3231
	3446	1007
TcR Vδ1/CD45RO	208	450
	59	106

Table A4.8 (continued):

b)

	CM		RBC		PfSE		PHA		PPD	
	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
cells in gate	147869	15471	154679	12405	141076	16327	130214	75555	166768	47546
	13869	3498	16344	1940	8751	1709	16365	16019	19176	13779
CD20	10286	1746	9969	1243	9457	2371	10325	15865	9737	7254
	1936	396	1924	365	1472	597	1691	2765	1780	3272
CD20/CD45RO	103	137	60	126	68	205	392	1738	90	529
	24	39	17	33	14	51	247	645	36	138
CD56	5982	1671	5418	842	6422	1812	5133	4761	9275	5373
	1514	680	1230	174	1523	293	1301	1626	2523	1265
CD56/CD45RO	342	197	246	164	195	326	745	1393	314	1164
	130	57	84	61	57	101	541	746	98	379
Total CD3	71392	8275	71022	5578	65621	8724	63810	55813	84395	30950
	11263	2444	10121	656	8353	902	11639	12456	14497	8908
CD45RA/CD3	34295	4481	33933	3051	29273	2788	21396	21704	34405	5854
	5891	1454	5450	428	4544	307	5010	5131	5224	1489
CD45RO/CD3	22916	5029	23164	3114	23434	6643	29904	36232	34062	26165
	3518	1478	2893	334	2522	786	5701	9675	6161	7557
TcRαβ/CD3	1118	776	1224	612	1094	1471	8853	18812	5239	7639
	459	202	546	188	396	401	4859	9058	3518	5077
TcRαβ/CD45RO	448	635	475	340	509	1347	2400	7792	2437	6488
	169	143	222	87	179	379	912	2070	1583	4021
TcRγδ/CD3	3962	826	3798	608	3124	962	3294	3744	4209	2320
	1003	259	964	109	910	173	955	1540	1039	550
TcRγδ/CD45RO	703	576	570	416	656	746	1086	2117	978	1668
	177	172	123	73	134	157	360	743	304	417
CD4/CD3	45955	6067	46913	4181	41470	6606	29444	29105	57756	26592
	7104	1722	6629	512	5683	737	5568	6636	10770	7992
CD4/CD45RO	19570	3989	20517	2852	18910	5442	14691	17601	30140	23260
	3156	1097	2862	367	2232	691	2378	3293	6751	6762
CD8/CD3	20159	3976	20024	2597	19682	4039	29784	34937	22342	6472
	3814	1115	3519	321	2880	693	6864	9496	4030	1525
CD8/CD45RO	2541	1346	2322	980	2562	1933	5881	9410	2827	3712
	628	247	570	144	504	335	1968	2936	684	807
TcR Vγ9/CD3	1976	618	2055	420	2112	668	4799	4844	2229	1461
	454	181	376	87	501	220	3242	3018	456	644
TcR Vγ9/CD45RO	423	362	476	284	535	518	1217	1725	478	1079
	99	105	143	90	122	208	501	667	166	461
TcR Vδ1/CD3	2440	372	2535	313	2413	732	2662	3257	3106	2025
	764	99	685	72	717	246	774	1506	788	603
TcR Vδ1/CD45RO	222	195	216	156	217	541	551	1726	540	1319
	103	48	98	48	85	266	289	807	259	440

Table A4.9: Summary data for percentages of cellular subsets stained on a) day 0, b) day 3 and c) day 7 for immune individuals (arithmetic mean and SEM indicated). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
% in gate	46.39	2.70
	5.30	0.50
CD20	4.35	15.83
	0.51	2.15
CD56	2.91	7.43
	0.42	1.18
CD56/CD3	1.22	1.49
	0.19	0.31
CD3	71.21	54.03
	3.14	3.46

b)

	RBC		PFSE	
	R1	R2	R1	R2
% in gate	38.78	1.92	37.63	2.33
	3.93	0.30	3.80	0.43
CD20	2.62	10.93	2.19	10.03
	0.33	1.37	0.34	1.50
CD56	2.08	3.61	2.56	2.72
	0.25	0.66	0.35	0.61
CD56/CD3	1.31	2.48	1.54	1.65
	0.18	0.58	0.29	0.53
CD3	74.33	70.58	71.73	70.72
	3.33	2.28	3.78	2.61

c)

	RBC		PfSE	
	R1	R2	R1	R2
% in gate	31.31	1.23	31.86	1.77
	3.96	0.33	3.74	0.35
CD20	1.81	9.89	2.02	8.84
	0.22	1.83	0.31	1.75
CD56	3.31	4.84	2.87	4.76
	1.12	1.25	0.68	0.65
CD56/CD3	2.56	2.72	1.89	2.12
	1.02	0.78	0.59	0.39
CD3	77.06	68.57	74.76	73.71
	3.70	3.87	4.08	2.74

Table A4.10: Summary data for total numbers of cells of cellular subsets stained on a) day 0, b) day 3 and c) day 7 for immune individuals (arithmetic mean and SEM indicated, cells are per 10^6 cells plated on day 0). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

	R1	R2
cells in gate	463889	27011
	52985	4974
CD20	19958	4033
	3194	849
CD56	13488	2272
	2511	554
CD56/CD3	5562	425
	1155	81
CD3	352601	15345
	53228	3660

b)

	RBC		PfSE	
DONOR	R1	R2	R1	R2
cells in gate	139322	7927	101654	7244
	23846	1972	18080	1858
CD20	4829	922	2852	827
	1289	215	758	227
CD56	3477	312	3126	307
	918	112	742	98
CD56/CD3	2114	210	1791	186
	531	78	464	63
CD3	111459	5984	77577	5519
	20369	1630	14513	1528

c)

	RBC		PfSE	
	R1	R2	R1	R2
cells in gate	81942	3397	103084	5067
	14158	1127	22471	1005
CD20	1721	347	2207	470
	474	121	516	127
CD56	2467	86	2973	341
	818	18	814	94
CD56/CD3	1953	51	1805	162
	723	11	528	45
CD3	67206	2586	83216	3826
	13396	946	20749	761

Table A4.11: Complete data for percentages of cellular subsets for each naïve individual on a) day 0, b) day 3 and c) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)
% in gate

DONOR	R1	R2
N3	82.8	8.5
N7	88.6	6.6
N8	84.3	7.1
N13	77.9	12.7
N14	86.0	8.2
N16	85.2	7.1
N17	84.4	7.9
N18	77.5	10.5
N19	87.4	6.2
N20	81.8	8.4

CD20

DONOR	R1	R2
N3	2.5	2.6
N7	5.3	0.9
N8	6.0	2.3
N13	5.4	1.7
N14	5.7	2.1
N16	2.5	1.3
N17	4.0	2.5
N18	6.5	2.3
N19	8.1	5.9
N20	6.3	3.5

CD56

DONOR	R1	R2
N3	1.3	7.4
N7	1.5	6.1
N8	2.4	5.2
N13	0.9	2.6
N14	2.0	12.4
N16	2.6	15.8
N17	0.5	3.5
N18	2.1	3.5
N19	0.7	3.8
N20	3.3	13.7

CD56/CD3

DONOR	R1	R2
N3	0.4	6.9
N7	0.5	4.5
N8	0.2	2.6
N13	0.1	1.7
N14	0.4	8.0
N16	0.4	10.0
N17	0.1	2.7
N18	0.4	1.1
N19	0.2	1.1
N20	0.9	8.6

Table A4.11a (continued):

Total CD3

DONOR	R1	R2
N3	83.7	67.2
N7	84.8	80.8
N8	82.4	68.0
N13	71.0	71.4
N14	85.3	63.1
N16	76.6	49.8
N17	74.0	70.2
N18	60.5	55.6
N19	80.9	55.8
N20	80.5	61.9

CD45RA/CD3

DONOR	R1	R2
N3	44.1	33.9
N7	60.4	44.2
N8	40.2	41.4
N13	40.3	31.7
N14	68.1	25.4
N16	58.2	24.6
N17	45.4	25.0
N18	36.1	33.6
N19	66.9	31.1
N20	52.9	29.3

CD45RO/CD3

DONOR	R1	R2
N3	66.3	66.7
N7	45.5	76.0
N8	67.1	66.5
N13	54.2	69.3
N14	51.9	60.6
N16	45.7	48.6
N17	58.2	69.3
N18	47.4	54.7
N19	52.7	54.4
N20	62.4	61.0

TcR$\alpha\beta$/CD3

DONOR	R1	R2
N3	71.6	8.6
N7	73.6	17.4
N8	65.8	22.5
N13	58.3	18.5
N14	77.5	31.0
N16	68.9	29.1
N17	64.9	19.8
N18	50.8	16.6
N19	70.3	24.4
N20	69.1	30.3

Table A4.11a (continued):

TcRγδ/CD3

DONOR	R1	R2
N3	1.7	1.0
N7	2.6	1.9
N8	10.2	6.0
N13	6.3	1.8
N14	2.3	3.8
N16	2.2	1.5
N17	4.0	1.8
N18	2.0	2.8
N19	2.4	1.3
N20	4.4	3.5

CD4/CD3

DONOR	R1	R2
N3	31.0	58.5
N7	57.6	71.0
N8	45.1	60.2
N13	31.8	57.3
N14	59.8	55.6
N16	51.2	50.7
N17	40.9	67.9
N18	26.2	44.6
N19	52.0	48.5
N20	39.5	52.5

CD8/CD3

DONOR	R1	R2
N3	34.4	38.9
N7	13.0	26.9
N8	20.8	37.0
N13	20.9	29.1
N14	14.0	30.0
N16	16.2	23.2
N17	26.6	13.2
N18	18.1	27.2
N19	12.8	25.0
N20	22.2	33.2

Table A4.11b:

b)
% in gate

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	80.2	1.1	78.9	2.9
N7	92.9	1.3	88.5	1.2
N8	87.3	1.6	85.2	2.0
N13	85.9	1.7	84.5	1.7
N14	89.4	2.0	85.9	2.0
N16	88.4	1.4	85.1	1.5
N17	86.6	1.6	76.3	2.3
N18	81.8	1.7	77.5	2.0
N19	89.1	1.1	85.2	1.7
N20	86.7	1.4	79.0	1.7

CD 20

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	0.4	1.8	0.6	2.5
N7	0.6	0.6	0.6	2.3
N8	1.3	2.3	0.9	2.5
N13	0.8	2.1	0.7	2.8
N14	1.8	1.7	1.7	3.5
N16	0.4	3.0	0.1	1.6
N17	2.4	7.6	1.8	8.9
N18	1.0	4.4	0.6	3.5
N19	0.2	2.4	0.3	1.0
N20	1.4	1.5	1.1	7.0

CD56

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	0.1	0.0	0.4	0.9
N7	0.0	0.0	0.1	1.2
N8	0.3	0.0	0.9	5.0
N13	0.2	0.4	0.5	0.0
N14	0.1	0.0	0.2	0.0
N16	0.1	0.0	0.3	2.6
N17	0.1	0.0	0.3	1.0
N18	0.7	0.0	1.2	3.5
N19	0.2	1.6	0.4	3.9
N20	0.2	0.0	0.4	3.7

CD56/CD3

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	0.1	0.0	0.2	0.0
N7	0.0	0.0	0.0	0.0
N8	0.0	0.0	0.0	0.8
N13	0.0	0.0	0.0	0.0
N14	0.0	0.0	0.0	0.0
N16	0.0	0.0	0.0	0.0
N17	0.0	0.0	0.1	0.0
N18	0.7	0.0	1.0	1.6
N19	0.0	0.8	0.0	0.0
N20	0.0	0.0	0.0	0.0

Table A4.11b (continued):

Total CD3

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	87.3	79.7	87.2	72.6
N7	88.9	86.2	85.9	80.1
N8	86.8	90.8	84.4	79.0
N13	71.5	70.0	71.8	67.9
N14	84.2	91.0	83.8	80.7
N16	80.5	75.2	79.1	74.2
N17	78.5	73.9	73.4	54.0
N18	71.1	79.8	71.2	72.8
N19	83.8	81.7	85.3	71.6
N20	85.6	87.3	84.0	70.6

Table A4.11c:

c)
% in gate

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	26.40	0.53	26.12	0.63	29.06	10.24	7.20	13.62	21.00	10.74
N7	38.60	0.08	43.02	0.24	47.60	7.20	7.16	19.62	52.18	2.36
N8	52.84	1.26	47.80	0.88	40.32	12.56	9.78	13.70	21.70	7.28
N13	35.64	0.19	32.34	0.20	45.40	3.28	17.38	13.04	27.06	9.24
N14	46.94	0.44	48.28	0.58	55.88	7.28	17.80	17.34	28.80	3.86
N16	37.52	0.20	41.40	0.20	46.48	9.14	9.32	19.28	20.28	22.56
N17	43.74	0.70	46.74	0.66	51.28	5.28	10.72	13.96	26.56	15.32
N18	30.28	0.82	31.20	0.05	39.32	6.08	11.18	17.34	34.94	0.76
N19	38.90	0.60	45.86	0.74	45.80	5.48	8.04	15.44	56.14	3.52
N20	39.50	0.36	30.53	0.31	34.82	13.00	11.22	17.82	15.76	25.54

CD20

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	2.05	1.45	1.85	5.75	2.44	3.78	2.61	2.23	1.21	2.42
N7	4.01	8.33	5.24	20.00	6.43	2.99	3.77	1.13	5.36	8.23
N8	3.09	13.33	2.83	10.00	9.89	5.36	5.58	2.59	5.76	2.67
N13	6.23	0.00	9.10	21.43	14.75	7.78	4.70	8.59	9.02	9.14
N14	10.28	35.29	9.06	11.43	17.25	8.57	1.90	2.51	8.74	9.50
N16	0.75	0.00	0.63	6.67	1.25	2.74	0.47	0.84	1.92	0.61
N17	6.98	39.02	5.77	31.25	8.28	17.49	0.58	1.24	4.88	2.87
N18	5.11	5.56	6.92	27.27	11.53	9.31	3.20	1.53	5.23	8.33
N19	11.93	18.18	9.87	12.50	22.99	14.77	5.70	5.77	17.57	15.60
N20	4.60	16.67	5.79	0.00	12.37	3.80	1.70	2.94	7.53	2.24

CD56

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	1.68	4.35	1.38	10.35	1.25	1.33	2.14	0.13	3.30	2.23
N7	0.76	ND	0.22	0.00	1.72	0.00	5.78	0.11	3.21	1.90
N8	1.15	6.67	1.24	0.00	3.39	1.25	5.58	0.15	5.58	5.34
N13	1.23	0.00	0.68	14.29	2.19	6.11	5.77	0.17	4.73	0.29
N14	1.07	0.00	1.78	2.86	2.98	0.57	3.27	0.00	4.31	2.71
N16	0.71	0.00	0.40	ND	7.40	1.47	5.84	0.31	7.39	0.61
N17	2.87	2.44	1.30	3.12	2.24	4.56	4.64	0.46	5.26	3.21
N18	7.57	0.00	7.66	0.00	7.79	6.47	5.94	0.24	9.23	0.00
N19	1.18	9.10	1.85	2.50	1.12	2.53	2.19	0.00	1.35	2.75
N20	1.60	0.00	1.89	0.00	3.24	0.79	3.56	0.36	5.06	1.32

CD56/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	1.17	1.45	1.23	2.30	0.07	0.22	0.48	0.13	0.00	0.37
N7	0.49	ND	0.09	0.00	0.33	0.00	0.75	0.11	1.02	0.63
N8	0.00	0.00	0.04	0.00	0.09	0.00	0.21	0.00	0.00	0.27
N13	0.66	0.00	0.34	14.29	0.82	1.67	0.43	0.00	1.51	0.00
N14	0.31	0.00	0.26	0.00	0.32	0.00	0.21	0.00	0.44	0.00
N16	0.38	0.00	0.23	ND	2.45	0.00	0.70	0.21	0.20	0.23
N17	2.25	2.44	1.02	3.12	0.54	0.76	0.39	0.31	0.30	0.80
N18	6.92	0.00	7.12	0.00	0.62	5.26	0.15	0.12	8.68	0.00
N19	0.79	4.55	1.48	0.00	0.30	0.00	0.44	0.00	0.66	0.00
N20	0.96	0.00	1.62	0.00	0.77	0.16	0.77	0.12	2.48	0.30

Table A4.11c (continued):

Total CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	93.14	54.46	88.28	47.50	91.64	85.60	88.06	95.84	91.46	86.79
N7	86.99	80.00	87.84	93.94	85.94	91.13	85.56	97.04	88.70	79.20
N8	91.92	85.19	90.51	52.38	84.00	91.42	79.52	96.33	87.03	87.22
N13	70.95	20.00	69.12	42.86	68.00	68.67	71.41	82.66	76.04	85.24
N14	79.80	47.37	82.83	76.93	74.76	83.46	87.23	95.06	80.97	81.45
N16	80.69	50.00	84.06	75.00	85.55	92.90	81.88	98.68	84.11	97.20
N17	87.03	71.43	85.88	54.76	83.63	69.05	83.06	95.59	85.88	88.20
N18	83.21	73.34	83.19	50.00	69.13	79.62	70.46	95.97	83.81	53.57
N19	76.02	21.06	83.48	67.92	70.31	83.50	89.55	87.13	79.78	78.97
N20	88.07	50.00	84.05	66.66	78.17	89.05	85.84	93.31	82.47	95.92

CD45RA/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	37.28	17.86	29.45	13.75	39.81	14.88	30.15	32.33	37.24	16.08
N7	50.44	43.33	52.32	27.27	52.82	20.12	38.58	40.57	58.37	32.89
N8	35.79	40.74	32.71	15.87	31.96	15.89	19.40	40.00	30.95	22.44
N13	24.48	20.00	22.05	28.57	24.73	17.47	24.35	31.54	39.52	11.48
N14	51.47	31.58	54.21	50.00	48.89	14.96	47.46	47.66	55.44	28.96
N16	48.53	33.33	53.77	75.00	55.04	16.39	34.59	24.92	48.26	9.95
N17	44.09	14.29	42.01	11.90	43.01	17.01	30.33	23.71	47.00	13.92
N18	42.59	33.33	41.86	50.00	34.14	9.81	21.89	26.67	39.25	21.43
N19	50.98	10.53	59.44	62.26	50.70	22.33	41.54	40.48	63.28	33.18
N20	42.48	25.00	34.04	16.67	37.16	16.22	24.01	33.56	45.93	16.90

CD45RO/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	33.01	41.07	29.13	25.00	29.81	77.80	42.09	55.95	32.20	80.62
N7	13.48	61.67	10.79	63.64	11.68	73.67	31.68	40.45	14.46	51.68
N8	35.13	35.19	34.58	36.51	32.93	85.71	50.86	60.76	33.90	74.15
N13	10.51	0.00	11.92	0.00	11.69	56.02	23.06	26.76	16.45	78.89
N14	8.09	5.26	8.98	23.08	9.27	71.39	16.27	23.70	9.55	66.52
N16	6.72	8.33	6.80	0.00	13.86	87.98	32.94	61.36	23.87	92.22
N17	26.31	50.00	22.87	23.81	23.87	56.46	32.22	50.15	25.25	79.58
N18	25.37	26.67	23.47	0.00	16.26	69.81	34.18	55.28	31.06	17.86
N19	5.77	10.53	5.19	5.66	5.88	62.46	13.68	13.72	7.52	50.47
N20	13.67	16.67	14.10	8.33	16.09	78.52	33.33	39.68	19.51	87.51

TcRαβ/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	42.54	35.04	41.22	31.06	47.06	66.25	46.80	72.71	52.36	70.54
N7	40.02	36.73	40.65	33.33	38.55	67.45	27.45	69.38	30.39	45.96
N8	30.12	31.37	36.24	16.22	38.16	68.11	15.07	60.04	41.43	70.47
N13	6.12	ND	7.03	ND	9.69	39.51	9.91	28.81	15.50	45.66
N14	25.59	25.00	22.10	24.00	20.16	54.02	33.94	61.69	29.90	57.00
N16	30.96	33.33	30.68	50.00	42.50	73.82	42.45	72.61	46.00	82.98
N17	28.78	23.40	29.43	3.85	27.25	38.38	31.76	63.61	38.02	71.34
N18	26.20	25.00	18.98	14.29	11.38	37.20	13.93	46.72	21.68	22.86
N19	18.49	11.11	19.54	35.00	17.08	42.75	31.41	52.25	16.67	33.33
N20	6.76	ND	5.48	ND	11.51	40.70	18.48	40.62	17.23	62.33

Table A4.11c (continued):

TcRγδ/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	3.46	4.90	2.67	3.85	1.21	14.56	2.58	16.81	4.23	12.72
N7	1.95	18.75	1.81	0.00	2.63	11.33	3.60	4.84	2.91	4.46
N8	5.02	6.12	5.70	2.50	5.58	12.47	8.85	7.07	5.76	20.13
N13	5.68	0.00	3.49	0.00	7.50	9.42	6.17	8.33	5.60	10.16
N14	1.96	0.00	2.88	0.00	2.67	8.70	2.21	4.93	2.78	19.89
N16	3.65	20.00	1.85	11.11	4.32	14.14	3.10	6.90	1.68	8.79
N17	6.26	0.00	4.26	4.19	ND	ND	4.19	6.20	2.49	10.17
N18	11.89	7.69	15.46	0.00	2.61	10.61	2.83	7.46	11.90	0.00
N19	3.97	0.00	4.35	0.00	2.55	8.85	4.10	6.90	3.97	9.34
N20	5.32	0.00	4.80	0.00	5.55	13.72	4.51	11.30	8.34	16.76

CD4/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	47.03	45.26	47.34	24.24	43.69	82.71	24.23	39.87	48.51	85.97
N7	64.83	40.82	64.68	26.67	64.96	83.28	52.51	77.88	65.78	66.46
N8	61.11	56.86	60.89	45.95	52.19	86.12	37.37	76.23	57.09	84.68
N13	27.22	33.33	26.76	16.67	30.95	75.12	36.64	40.89	39.10	80.92
N14	55.04	43.75	54.13	36.00	51.52	77.01	62.06	74.46	55.29	80.50
N16	48.67	33.33	56.42	50.00	59.87	90.55	60.14	73.05	58.91	96.53
N17	55.73	34.04	50.54	23.08	46.51	61.28	38.56	63.92	53.15	81.76
N18	53.23	25.00	49.13	28.57	37.59	73.72	33.28	64.52	51.53	40.00
N19	52.19	11.11	58.34	52.50	47.90	78.81	49.75	51.26	54.65	54.85
N20	45.96	26.32	41.51	14.29	40.62	85.26	47.32	54.12	46.57	94.69

CD8/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	35.20	14.69	33.31	13.46	38.83	5.22	57.47	57.71	39.03	7.46
N7	15.68	18.75	15.39	16.67	12.28	0.65	18.88	21.01	17.42	13.39
N8	16.00	14.29	13.58	12.50	14.52	1.46	18.58	18.65	15.87	3.57
N13	9.90	0.00	6.66	0.00	7.18	1.45	19.23	36.64	13.89	1.22
N14	7.61	0.00	6.35	11.11	5.99	2.56	16.63	18.03	11.20	7.53
N16	11.52	20.00	17.24	11.11	17.86	1.64	20.53	29.25	14.76	0.70
N17	3.79	0.00	2.52	1.57	ND	ND	10.12	15.90	0.88	0.42
N18	26.74	7.69	25.66	0.00	18.60	4.90	29.51	34.93	30.44	21.95
N19	9.51	6.67	9.91	4.76	8.74	1.15	25.68	31.14	14.08	8.24
N20	16.07	7.69	14.27	14.29	20.69	1.10	23.57	39.92	19.58	1.06

Table A4.12: Complete data for total numbers of cells (per 10^6 cells plated on day 0) of cellular subsets for each naïve individual on a) day 0, b) day 3 and c) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)
cells in gate

DONOR	R1	R2
N3	828200	85000
N7	886300	65900
N8	842800	70800
N13	779200	127400
N14	859800	82200
N16	852000	70900
N17	844000	78800
N18	774800	105400
N19	873900	62300
N20	817900	84200

CD20

DONOR	R1	R2
N3	20539	2176
N7	47240	606
N8	50821	1607
N13	42077	2217
N14	48837	1726
N16	21470	936
N17	33422	1954
N18	50362	2382
N19	70699	3645
N20	51446	2913

CD56

DONOR	R1	R2
N3	11098	6316
N7	13560	3994
N8	20227	3667
N13	6623	3274
N14	16938	10176
N16	22408	11216
N17	4389	2734
N18	16193	3710
N19	6292	2367
N20	26909	11552

CD56/CD3

DONOR	R1	R2
N3	3644	5823
N7	4609	2952
N8	1854	1834
N13	1091	2217
N14	3525	6543
N16	3323	7062
N17	506	2151
N18	3099	1191
N19	1573	692
N20	7606	7233

Table A4.12a (continued):

Total CD3

DONOR	R1	R2
N3	693038	57078
N7	751848	53241
N8	694720	48137
N13	553310	90925
N14	732980	51868
N16	652291	35287
N17	624729	55278
N18	469064	58581
N19	706898	34732
N20	658164	52128

CD45RA/CD3

DONOR	R1	R2
N3	365071	28773
N7	535148	29115
N8	338468	29276
N13	313862	40360
N14	585868	20846
N16	496120	17427
N17	383429	19676
N18	279315	35435
N19	584901	19350
N20	432587	24637

CD45RO/CD3

DONOR	R1	R2
N3	548683	56695
N7	403089	50051
N8	565350	47054
N13	422015	88250
N14	446580	49813
N16	389279	34422
N17	490955	54640
N18	366868	57685
N19	460371	33860
N20	510451	51387

TcRαβ/CD3

DONOR	R1	R2
N3	592660	7285
N7	651962	11493
N8	554310	15937
N13	454196	23505
N14	666603	25507
N16	586943	20618
N17	547587	15587
N18	393521	17507
N19	614526	15214
N20	564924	25471

Table A4.12a (continued):

TcRγδ/CD3

DONOR	R1	R2
N3	13914	833
N7	23132	1219
N8	85713	4213
N13	49168	2268
N14	19603	3148
N16	18914	1035
N17	33844	1442
N18	15573	2930
N19	20711	835
N20	35906	2930

CD4/CD3

DONOR	R1	R2
N3	257073	49742
N7	510243	46815
N8	379934	42607
N13	247786	72987
N14	513902	45720
N16	436139	35932
N17	345112	53497
N18	202688	47051
N19	454166	30228
N20	323398	44230

CD8/CD3

DONOR	R1	R2
N3	2.851	0.330
N7	1.149	0.177
N8	1.749	0.262
N13	1.629	0.370
N14	1.199	0.246
N16	1.379	0.164
N17	2.248	0.104
N18	1.402	0.286
N19	1.120	0.156
N20	1.817	0.280

Table A4.12b:

b)
cells in gate

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	421050	5828	406438	14987
N7	640803	8625	587052	10557
N8	449338	8292	524042	12546
N13	377872	7612	464640	9130
N14	625450	13790	850509	20196
N16	618660	9870	587052	10557
N17	402504	7626	400523	11970
N18	347565	7225	515176	13500
N19	748188	9324	617773	12470
N20	381392	5940	395200	8700

CD 20

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	1516	102	2520	373
N7	3588	54	704	167
N8	5752	191	4769	316
N13	3023	157	3113	257
N14	11258	237	14714	701
N16	2227	299	704	167
N17	9660	580	7330	1067
N18	3510	316	3297	478
N19	1347	222	1977	122
N20	5492	89	4387	605

CD56

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	547	0	1666	139
N7	192	0	1644	278
N8	1528	0	4559	632
N13	907	31	2184	0
N14	876	0	1871	0
N16	742	0	1644	278
N17	564	0	1282	119
N18	2468	0	6337	478
N19	1197	147	2286	489
N20	801	0	1541	325

CD56/CD3

	RBC		PFSE	
DONOR	R1	R2	R1	R2
N3	337	0	935	0
N7	64	0	59	0
N8	45	0	210	105
N13	76	0	186	0
N14	125	0	85	0
N16	0	0	59	0
N17	40	0	320	0
N18	2259	0	5049	212
N19	75	74	124	0
N20	76	0	0	0

Table A4.12b (continued):

Total CD3

	RBC		PFSE	
DONOR	**R1**	**R2**	**R1**	**R2**
N3	367577	4642	354536	10879
N7	569353	7431	464476	7834
N8	389845	7529	442291	9910
N13	270254	5325	333426	6197
N14	526879	12542	712301	16296
N16	497959	7417	464476	7834
N17	316086	5636	294064	6459
N18	246980	5763	366908	9832
N19	626832	7621	526898	8925
N20	326586	5186	331849	6141

Table A4.12c:

c)
cells in gate

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	145200	2915	90114	2174	130770	46080	44640	84444	92400	47256
N7	196860	408	225855	1260	328440	49680	49404	135378	135668	6136
N8	298546	7119	198370	3652	348768	108644	68460	95900	151900	50960
N13	83754	447	84084	520	231540	16728	59961	44988	63591	21714
N14	206536	1936	386240	4640	477774	62244	110360	107508	174240	23353
N16	150080	800	240120	1160	339304	66722	37280	77120	97344	108288
N17	168399	2695	102828	1452	253836	26136	68072	88646	135456	78132
N18	62074	1681	56160	90	98300	15200	86086	133518	120543	2622
N19	225620	3480	587008	9472	359530	43018	104118	199948	286314	17952
N20	86900	792	109908	1116	125352	46800	172788	274428	110320	178780

CD20

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	2977	42	1667	125	3191	1742	1165	1883	1118	1144
N7	7894	34	11835	252	21119	1485	1863	1530	7272	505
N8	9225	949	5614	365	34493	5823	3820	2484	8749	1361
N13	5218	0	7652	111	34152	1301	2818	3864	5736	1985
N14	21232	683	34993	530	82416	5334	2097	2698	15229	2219
N16	1126	0	1513	77	4241	1828	175	648	1869	661
N17	11754	1052	5933	454	21018	4571	395	1099	6610	2242
N18	3172	93	3886	25	11334	1415	2755	2043	6304	218
N19	26916	633	57938	1184	82656	6354	5935	11537	50305	2801
N20	3997	132	6364	0	15506	1778	2937	8068	8307	4005

CD56

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	2439	127	1244	225	1635	613	955	110	3049	1054
N7	1496	ND	497	0	5649	0	2856	149	4355	117
N8	3433	475	2460	0	11823	1358	3820	144	8476	2721
N13	1030	0	572	74	5071	1022	3460	76	3008	63
N14	2210	0	6875	133	14238	355	3609	0	7510	633
N16	1066	0	960	ND	25108	981	2177	239	7194	661
N17	4833	66	1337	45	5686	1192	3159	408	7125	2508
N18	4699	0	4302	0	7658	983	5114	320	11126	0
N19	2662	317	10860	237	4027	1088	2280	0	3865	494
N20	1390	0	2077	0	4061	370	6151	988	5582	2360

CD56/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	1699	42	1108	50	92	101	214	110	0	175
N7	965	ND	203	0	1084	0	371	149	1384	39
N8	0	0	79	0	314	0	144	0	0	138
N13	553	0	286	74	1899	279	258	0	960	0
N14	640	0	1004	0	1529	0	232	0	767	0
N16	570	0	552	ND	8313	0	261	162	195	249
N17	3789	66	1049	45	1371	199	265	275	406	625
N18	4296	0	3999	0	609	800	129	160	10463	0
N19	1782	158	8688	0	1079	0	458	0	1890	0
N20	834	0	1781	0	965	75	1330	329	2736	536

Table A4.12c (continued):

Total CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	135239	1588	79553	1032	119838	39444	39310	80931	84509	41013
N7	171249	326	198391	1184	282261	45273	42270	131371	120338	4860
N8	274423	6065	179545	1913	292965	99322	54439	92380	132199	44447
N13	59423	89	58119	223	157447	11487	42818	37187	48355	18509
N14	164816	917	319923	3570	357184	51949	96267	102197	141082	19021
N16	121100	400	201845	870	290275	61985	30525	76102	81876	105256
N17	146558	1925	88309	795	212283	18047	56541	84737	116330	68912
N18	51652	1233	46720	45	67955	12102	60656	128137	101027	1405
N19	171516	733	490034	6433	252786	35920	93238	174215	228421	14177
N20	76533	396	92378	744	97988	41675	148321	256069	90981	171486

CD45RA/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	54131	521	26539	299	52060	6857	13459	27301	34410	7599
N7	99296	177	118167	344	173482	9996	19060	54923	79189	2018
N8	106850	2900	64887	580	111466	17264	13281	38360	47013	11435
N13	20503	89	18541	149	57260	2922	14601	14189	25131	2493
N14	106304	611	209381	2320	233584	9312	52377	51238	96599	6763
N16	72834	267	129113	870	186753	10936	12895	19218	46978	10775
N17	74247	385	43198	173	109175	4446	20646	21018	63664	10876
N18	26437	560	23509	45	33560	1491	18844	35609	47313	562
N19	115021	366	348918	5897	182282	9606	43251	80939	181179	5956
N20	36915	198	37413	186	46581	7591	41486	92098	50670	30214

CD45RO/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	47931	1197	26250	543	38983	35850	18789	47246	29753	38098
N7	26537	252	24370	802	38362	36599	15651	54760	19618	3171
N8	104879	2505	68596	1333	114849	93119	34819	58269	51494	37787
N13	8803	0	10023	0	27067	9371	13827	12039	10461	17130
N14	16709	102	34684	1071	44290	44436	17956	25479	16640	15534
N16	10085	67	16328	0	47028	58702	12280	47321	23236	99863
N17	44306	1348	23517	346	60591	14756	21933	44456	34203	62177
N18	15748	448	13181	0	15984	10611	29424	73809	37441	468
N19	13018	366	30466	536	21140	26869	14243	27433	21531	9060
N20	11879	132	15497	93	20169	36747	57590	108893	21523	156450

TcRαβ/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	61768	1021	37145	675	61540	30528	20892	61399	48381	33334
N7	78783	150	91810	420	126614	33509	13561	93925	41230	2820
N8	89922	2233	71889	592	133090	73997	10317	57578	62932	35912
N13	5126	ND	5911	ND	22436	6609	5942	12961	9857	9915
N14	52853	484	85359	1114	96319	33624	37456	66322	52098	13311
N16	46465	267	73669	580	144204	49254	15825	55997	44778	89857
N17	48465	631	30262	56	69170	10031	21620	56388	51500	55739
N18	16263	420	10659	13	11187	5654	11992	62380	26134	599
N19	41717	387	114701	3315	61408	18390	32703	104473	47729	5983
N20	5874	ND	6023	ND	14428	19048	31931	111473	19008	111434

Table A4.12c (continued):

TcRγδ/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	5024	143	2406	84	1582	6709	1152	14195	3909	6011
N7	3839	77	4088	0	8638	5629	1779	6552	3948	274
N8	14987	436	11307	91	19461	13548	6059	6780	8749	10258
N13	4757	0	2935	0	17366	1576	3700	3748	3561	2206
N14	4048	0	11124	0	12757	5415	2439	5300	4844	4645
N16	5478	160	4442	129	14658	9434	1156	5321	1635	9519
N17	10542	0	4380	61	ND	ND	2852	5496	3373	7946
N18	7381	129	8682	0	2566	1613	2436	9960	14345	0
N19	8957	0	25535	0	9168	3807	4269	13796	11367	1677
N20	4623	0	5276	0	6957	6421	7793	31010	9201	29964

CD4/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	68288	1319	42660	527	57133	38113	10816	33668	44823	40626
N7	127624	167	146083	336	213355	41374	25942	105432	89242	4078
N8	182441	4048	120787	1678	182022	93564	25584	73105	86720	43153
N13	22798	149	22501	87	71662	12566	21970	18396	24864	17571
N14	113677	847	209072	1670	246149	47934	68489	80050	96337	18799
N16	73044	267	135476	580	203141	60417	22420	56336	57345	104530
N17	93849	917	51969	335	118059	16016	26249	56663	71995	63881
N18	33042	420	27591	26	36951	11205	28649	86146	62116	1049
N19	117751	387	342460	4973	172215	33902	51799	102493	156471	9847
N20	39939	208	45623	159	50918	39902	81763	148520	51376	169287

CD8/CD3

	CM		RBC		PFSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
N3	51110	428	30017	293	50778	2405	25655	48733	36064	3525
N7	30868	77	34759	210	40332	323	9327	28443	23633	822
N8	47767	1017	26939	457	50641	1586	12720	17885	24107	1819
N13	8292	0	5600	0	16625	243	11531	16484	8833	265
N14	15717	0	24526	516	28619	1593	18353	19384	19515	1758
N16	17289	160	41397	129	60600	1094	7654	22558	14368	758
N17	6382	0	2591	23	ND	ND	6889	14095	1192	328
N18	16599	129	14411	0	18284	745	25404	46638	36693	576
N19	21456	232	58172	451	31423	495	26738	62264	40313	1479
N20	13965	61	15684	159	25935	515	40726	109552	21601	1895

Table A4.13: Complete data for percentages of cellular subsets for each exposed individual on a) day 0 and b) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)
% in gate

DONOR	R1	R2
E1	35.64	38.91
E2	9.08	10.23
E3	34.27	4.62
E4	26.61	2.03
E5	27.65	1.54
E6	52.96	5.28
E7	55.60	7.50
E8	47.75	6.38
E9	38.94	11.42
E10	22.58	5.93
E11	43.83	19.10
E12	46.73	3.19
E14	48.54	1.79
E15	70.88	4.78
E16	65.77	4.80
E17	64.64	5.25
E18	62.15	5.07
E20	50.81	2.29

CD20

DONOR	R1	R2
E1	15.23	8.31
E2	4.36	17.39
E3	9.27	6.99
E4	7.85	12.00
E5	11.73	14.96
E6	5.07	11.81
E7	11.88	9.72
E8	1.36	7.66
E9	9.86	11.47
E10	11.50	12.50
E11	24.72	40.00
E12	10.66	14.20
E14	13.41	19.50
E15	8.70	16.88
E16	8.80	7.84
E17	6.94	44.09
E18	14.88	34.55
E20	4.61	10.91

CD20/CD45RO

DONOR	R1	R2
E1	0.17	0.30
E2	0.00	0.18
E3	0.68	0.97
E4	0.00	0.00
E5	0.00	0.39
E6	0.00	0.00
E7	0.07	0.47
E8	0.07	0.35
E9	0.00	0.34
E10	0.00	0.00
E11	0.03	0.00
E12	0.00	0.97
E14	0.02	0.83
E15	0.07	0.18
E16	0.03	0.17
E17	0.02	1.08
E18	0.02	0.91
E20	0.04	1.21

Table A4.13a (continued):

CD56

DONOR	R1	R2
E1	0.57	1.82
E2	0.00	2.93
E3	7.88	15.92
E4	0.37	0.89
E5	0.38	1.96
E6	0.24	0.79
E7	0.37	2.66
E8	2.76	13.46
E9	0.40	3.21
E10	0.38	2.81
E11	0.10	0.36
E12	0.26	1.29
E14	0.16	2.49
E15	0.25	1.27
E16	0.53	1.53
E17	0.40	0.86
E18	0.25	0.60
E20	0.17	1.82

CD56/CD45RO

DONOR	R1	R2
E1	0.04	0.12
E2	0.00	0.00
E3	7.32	14.37
E4	0.04	0.00
E5	0.00	0.39
E6	0.00	0.00
E7	0.02	0.31
E8	0.22	1.16
E9	0.00	0.17
E10	0.00	0.31
E11	0.05	0.18
E12	0.04	0.32
E14	0.02	0.83
E15	0.03	0.36
E16	0.01	0.17
E17	0.05	0.43
E18	0.03	0.30
E20	0.00	0.61

Total CD3

DONOR	R1	R2
E1	74.04	76.98
E2	49.39	64.16
E3	64.71	59.14
E4	62.86	52.80
E5	53.17	66.76
E6	61.80	61.39
E7	64.86	59.20
E8	63.51	40.42
E9	47.10	64.09
E10	55.90	59.94
E11	55.50	45.09
E12	59.68	49.28
E14	49.77	41.96
E15	59.42	44.38
E16	60.56	31.72
E17	74.06	33.84
E18	62.98	39.90
E20	28.53	54.31

Table A4.13a (continued):

CD45RA/CD3

DONOR	R1	R2
E1	45.06	39.85
E2	25.38	19.47
E3	26.98	18.28
E4	34.16	24.80
E5	33.35	33.82
E6	42.99	34.74
E7	36.20	25.61
E8	36.29	18.09
E9	25.15	37.11
E10	30.04	24.46
E11	29.15	22.37
E12	15.77	13.33
E14	32.09	21.77
E15	28.39	17.95
E16	35.60	13.00
E17	45.28	12.69
E18	23.90	19.95
E20	20.22	37.07

CD45RO/CD3

DONOR	R1	R2
E1	26.88	40.82
E2	19.64	44.25
E3	31.88	40.32
E4	22.84	30.40
E5	14.96	35.29
E6	13.95	32.90
E7	22.10	31.40
E8	19.91	19.40
E9	15.90	39.20
E10	22.48	41.59
E11	19.55	26.78
E12	35.68	40.58
E14	11.68	19.56
E15	27.21	34.12
E16	15.37	15.64
E17	17.28	16.07
E18	27.10	28.43
E20	4.47	19.40

TcRαβ/CD3

DONOR	R1	R2
E1	2.12	6.28
E2	0.49	1.48
E3	1.01	3.33
E4	2.07	5.18
E5	3.45	5.85
E6	4.83	9.52
E7	2.96	3.65
E8	5.08	6.82
E9	3.68	7.79
E10	0.68	0.28
E11	0.03	0.33
E12	0.02	1.70
E14	0.00	0.39
E15	0.13	1.03
E16	0.01	0.19
E17	0.12	1.54
E18	0.06	0.75
E20	0.02	1.16

Table A4.13a (continued):

TcRαβ/CD45RO

DONOR	R1	R2
E1	0.32	1.36
E2	0.18	0.49
E3	0.39	2.22
E4	0.08	1.59
E5	0.16	1.46
E6	0.77	2.67
E7	0.43	0.66
E8	0.25	1.04
E9	0.62	3.18
E10	0.25	0.28
E11	0.00	0.67
E12	0.00	1.42
E14	0.00	0.39
E15	0.09	0.77
E16	0.00	0.19
E17	0.02	1.32
E18	0.00	0.57
E20	0.02	1.16

TcRγδ/CD3

DONOR	R1	R2
E1	3.39	7.60
E2	0.86	7.32
E3	0.92	3.46
E4	2.57	2.45
E5	1.86	2.94
E6	3.90	11.75
E7	2.45	7.17
E8	4.82	7.47
E9	2.90	8.19
E10	0.96	2.38
E11	7.01	7.38
E12	3.07	4.88
E14	1.22	1.11
E15	1.78	3.19
E16	1.92	2.63
E17	4.33	5.74
E18	11.69	5.83
E20	2.27	10.07

TcRγδ/CD45RO

DONOR	R1	R2
E1	0.85	1.75
E2	0.31	4.88
E3	0.37	1.57
E4	0.24	1.05
E5	0.15	1.47
E6	0.51	5.97
E7	0.37	2.12
E8	0.35	0.96
E9	0.15	4.71
E10	0.33	2.12
E11	0.46	2.62
E12	0.86	3.08
E14	0.11	0.00
E15	0.37	2.03
E16	0.19	0.75
E17	0.42	2.98
E18	0.13	0.45
E20	0.04	3.36

Table A4.13a (continued):

CD4/CD3

DONOR	R1	R2
E1	44.13	45.59
E2	30.53	53.63
E3	47.84	50.00
E4	40.55	36.33
E5	35.22	40.00
E6	23.04	29.09
E7	2.59	5.57
E8	35.77	21.04
E9	21.61	31.29
E10	35.56	42.66
E11	25.17	27.91
E12	36.21	42.55
E14	27.82	34.36
E15	30.94	44.94
E16	37.46	45.52
E17	47.52	31.10
E18	31.16	42.73
E20	15.74	33.49

CD4/CD45RO

DONOR	R1	R2
E1	15.59	23.97
E2	8.59	26.26
E3	18.52	31.05
E4	11.45	22.66
E5	9.48	19.68
E6	4.84	14.18
E7	0.38	1.91
E8	8.73	9.45
E9	11.51	24.10
E10	15.17	26.57
E11	11.52	9.51
E12	23.20	32.22
E14	5.80	12.74
E15	12.40	33.93
E16	7.93	14.41
E17	9.46	11.54
E18	14.79	24.86
E20	2.59	11.63

CD8/CD3

DONOR	R1	R2
E1	19.80	31.70
E2	13.10	41.18
E3	10.95	32.79
E4	14.75	27.01
E5	15.47	30.91
E6	20.74	42.40
E7	39.86	35.28
E8	18.23	19.39
E9	14.84	35.34
E10	13.06	28.79
E11	15.32	29.19
E12	7.20	21.77
E14	9.70	34.44
E15	16.13	22.47
E16	15.95	23.99
E17	16.07	23.33
E18	12.38	17.66
E20	7.39	23.08

Table A4.13a (continued):

CD8/CD45RO

DONOR	R1	R2
E1	1.17	3.59
E2	1.35	4.41
E3	0.36	4.37
E4	0.80	5.84
E5	0.49	2.69
E6	1.26	6.01
E7	15.83	22.45
E8	0.89	1.50
E9	1.13	8.10
E10	1.61	6.44
E11	0.53	1.52
E12	1.29	4.30
E14	0.36	0.74
E15	3.51	4.25
E16	0.60	1.06
E17	0.92	2.78
E18	2.34	4.37
E20	0.04	1.81

TcR Vγ9/CD3

DONOR	R1	R2
E1	3.02	6.56
E2	0.90	0.91
E3	8.93	13.94
E4	2.04	2.69
E5	0.93	3.46
E6	2.09	4.35
E7	18.52	25.53
E8	2.38	2.13
E9	2.11	4.65
E10	0.72	1.13
E11	5.50	2.97
E12	3.57	2.97
E14	0.76	0.77
E15	0.87	1.05
E16	1.37	1.83
E17	4.71	6.03
E18	6.40	2.13
E20	0.36	1.14

TcR Vγ9/CD45RO

DONOR	R1	R2
E1	0.11	1.75
E2	0.09	0.00
E3	1.74	4.24
E4	0.11	1.35
E5	0.04	0.69
E6	0.24	2.57
E7	0.87	2.44
E8	0.07	0.51
E9	0.38	1.83
E10	0.24	0.75
E11	0.15	0.93
E12	0.16	0.37
E14	0.00	0.38
E15	0.21	0.42
E16	0.04	0.17
E17	0.06	3.02
E18	0.13	1.07
E20	0.00	0.00

Table A4.13a (continued):

TcR Vδ1/CD3

DONOR	R1	R2
E1	2.46	3.39
E2	0.78	0.68
E3	0.12	2.08
E4	2.23	1.46
E5	2.66	6.93
E6	5.19	5.56
E7	1.88	4.55
E8	7.72	8.11
E9	3.47	6.82
E10	0.39	0.00
E11	6.84	7.34
E12	0.49	1.06
E14	0.72	1.12
E15	0.54	1.24
E16	1.18	1.55
E17	1.62	2.70
E18	8.68	5.59
E20	2.96	10.86

TcR Vδ1/CD45RO

DONOR	R1	R2
E1	0.06	0.15
E2	0.05	0.00
E3	0.06	0.00
E4	0.00	0.73
E5	0.00	0.00
E6	0.00	0.51
E7	0.13	0.76
E8	0.17	0.53
E9	0.03	1.00
E10	0.00	0.00
E11	0.03	0.56
E12	0.02	1.41
E14	0.06	0.56
E15	0.04	0.35
E16	0.00	0.58
E17	0.05	1.87
E18	0.08	2.93
E20	0.00	2.29

Table A4.13b:

b)
% in gate

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	41.33	4.85	37.56	3.40	41.24	7.24	36.27	22.76	39.27	11.02
E2	32.60	3.79	33.01	2.48	33.44	2.47	26.18	18.10	33.70	2.95
E3	40.49	7.34	35.20	0.88	37.08	1.77	28.16	9.41	32.21	8.99
E4	33.50	1.17	34.20	1.06	34.48	1.68	29.63	8.40	33.51	1.83
E5	37.45	0.68	36.21	1.82	35.34	2.17	27.71	12.68	35.93	1.77
E6	48.25	2.04	47.35	2.88	47.09	2.88	42.56	20.29	39.63	6.68
E7	40.02	1.97	48.97	4.02	47.06	4.94	35.20	27.97	45.51	6.73
E8	40.56	17.80	54.97	5.43	51.08	5.78	32.88	27.18	44.20	11.63
E9	32.38	2.78	32.46	2.02	32.39	4.29	23.95	4.69	31.10	3.68
E10	33.46	1.89	31.73	1.63	30.08	5.13	24.79	8.35	31.39	2.79
E11	35.25	5.46	34.01	7.06	35.23	7.68	22.85	24.23	32.58	14.30
E12	31.94	3.73	31.48	3.84	33.41	4.91	26.14	17.38	31.11	10.49
E14	27.43	0.47	31.68	1.87	33.45	3.38	31.68	10.24	31.40	4.08
E15	47.84	2.00	48.24	1.60	42.41	6.00	27.65	18.11	39.46	9.67
E16	39.39	4.81	42.56	4.50	43.14	5.32	29.22	28.81	38.94	6.01
E17	56.42	3.52	60.03	3.98	56.34	3.50	35.18	18.77	58.88	4.40
E18	37.16	6.20	35.08	6.44	32.20	6.84	25.97	9.16	34.63	7.10
E20	46.71	3.93	48.22	4.02	37.18	7.55	30.13	14.72	31.90	18.63

CD20

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	10.65	13.44	10.99	11.44	9.35	11.63	13.21	26.69	6.49	9.64
E2	3.27	10.00	1.77	4.39	3.97	16.39	4.95	21.82	4.01	11.26
E3	3.53	10.37	2.89	10.17	3.21	10.06	5.16	23.48	2.38	9.22
E4	5.26	11.85	3.58	11.32	3.24	14.05	5.36	19.45	3.16	13.33
E5	4.78	8.93	5.07	5.82	5.55	9.68	6.05	15.44	6.01	13.49
E6	3.69	10.57	3.13	7.40	3.94	10.49	4.15	11.43	3.81	6.61
E7	9.49	5.29	8.89	4.99	10.48	8.42	8.16	18.51	7.90	8.81
E8	6.58	8.96	7.47	13.51	6.87	13.60	5.41	17.14	4.79	8.24
E9	5.03	13.64	3.85	8.20	4.31	14.20	0.00	0.22	2.74	11.08
E10	3.85	13.29	2.96	8.93	3.78	15.99	4.55	22.27	3.18	15.20
E11	16.99	31.83	15.35	29.03	10.31	22.93	15.21	25.39	14.65	17.41
E12	4.99	12.32	4.35	14.23	4.38	20.52	23.13	67.45	3.24	6.08
E14	2.91	0.81	2.31	5.47	3.77	15.42	7.52	18.63	3.07	8.26
E15	7.48	20.45	6.86	16.04	5.30	9.61	7.29	27.95	3.20	7.06
E16	4.46	6.10	4.57	2.82	4.43	4.14	4.37	15.23	7.72	29.05
E17	5.83	9.83	7.66	7.08	12.63	5.09	9.51	15.95	12.19	24.30
E18	5.04	1.72	6.87	0.91	7.86	4.28	18.10	30.05	9.91	17.87
E20	22.79	19.70	13.61	20.62	14.83	39.53	18.93	44.06	6.38	25.70

CD20/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.11	1.98	0.00	4.11	0.08	1.94	0.06	2.68	0.06	1.98
E2	0.00	1.33	0.06	0.88	0.06	2.46	0.17	3.19	0.03	0.87
E3	0.03	1.22	0.02	4.24	0.00	1.18	0.00	3.20	0.03	2.94
E4	0.03	1.32	0.03	0.94	0.06	0.83	0.00	1.51	0.03	1.43
E5	0.03	0.89	0.00	1.94	0.03	1.94	0.12	2.50	0.00	2.38
E6	0.00	0.81	0.02	1.23	0.04	0.00	0.09	0.51	0.00	0.27
E7	0.05	0.79	0.15	0.83	0.07	0.86	0.31	3.21	0.02	1.36
E8	0.04	0.90	0.00	1.08	0.00	1.87	0.05	1.40	0.02	0.86
E9	0.03	0.00	0.06	0.00	0.06	1.45	0.00	0.00	0.00	0.81
E10	0.06	0.00	0.00	0.00	0.00	0.00	0.04	0.74	0.03	0.00
E11	0.08	0.60	0.09	1.07	0.12	3.60	0.03	1.04	0.00	0.95
E12	0.12	0.70	0.03	1.50	0.04	1.78	8.07	32.23	0.03	0.40
E14	0.03	0.00	0.00	0.68	0.00	0.42	0.03	1.58	0.00	1.14
E15	0.04	2.27	0.00	1.60	0.02	1.01	0.07	3.15	0.00	0.51
E16	0.09	1.38	0.02	0.00	0.07	0.17	0.00	0.11	0.24	3.73
E17	0.12	0.58	0.04	0.39	0.09	0.85	0.28	0.55	0.05	2.23
E18	0.24	0.31	0.08	0.13	0.11	0.27	0.27	1.69	0.24	2.75
E20	0.16	0.00	0.06	1.72	0.00	1.83	0.33	1.44	0.09	0.34

Table A4.13b (continued):

CD56

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	3.51	14.82	2.69	6.74	4.39	15.89	2.57	2.33	5.55	11.36
E2	1.21	2.66	0.96	3.51	1.16	21.31	4.58	8.17	1.97	15.58
E3	3.82	20.73	3.57	8.47	4.94	23.08	2.20	11.10	4.58	10.64
E4	2.66	15.79	2.42	15.09	1.46	9.09	1.04	2.64	2.42	14.28
E5	2.22	9.82	1.90	9.71	2.17	12.90	1.44	2.08	2.83	15.88
E6	6.78	14.64	6.73	20.99	5.99	11.99	3.58	2.69	8.15	11.61
E7	3.47	5.03	4.44	15.23	4.95	6.91	2.78	3.17	7.99	11.51
E8	16.85	17.91	18.47	20.54	17.29	24.49	12.36	3.42	22.20	29.50
E9	2.65	7.57	3.06	4.92	4.74	13.33	0.00	0.22	3.84	8.65
E10	2.25	6.29	1.92	3.58	3.49	9.30	1.10	1.98	2.96	6.37
E11	0.45	1.35	0.29	2.53	0.65	1.50	0.73	3.45	0.89	4.54
E12	1.36	6.34	1.35	3.75	2.47	6.54	39.84	68.44	3.05	13.54
E14	0.15	0.00	0.26	2.74	1.86	10.00	0.42	1.46	1.99	12.82
E15	4.83	16.48	4.48	17.64	7.81	19.89	7.06	6.20	8.89	23.62
E16	2.97	5.11	2.88	3.39	2.35	6.90	2.68	6.49	3.29	17.37
E17	5.47	6.36	4.95	7.87	4.99	9.75	4.00	2.89	6.86	15.09
E18	3.24	0.94	2.95	0.26	4.54	2.54	5.30	12.38	6.03	11.00
E20	0.97	4.48	0.70	3.09	1.30	13.87	1.98	4.25	1.44	3.80

CD56/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.29	1.38	0.10	1.17	0.03	2.71	0.06	0.79	0.17	2.25
E2	0.15	1.33	0.03	0.88	0.09	4.10	0.00	1.66	0.06	2.16
E3	0.16	1.83	0.10	0.00	0.11	8.88	0.10	3.42	0.08	3.65
E4	0.12	1.32	0.11	2.83	0.09	0.83	0.03	0.88	0.09	3.33
E5	0.00	0.00	0.03	0.97	0.06	0.00	0.15	1.04	0.09	1.59
E6	0.69	4.07	0.72	8.64	0.41	2.25	0.48	0.42	0.65	4.59
E7	0.17	0.53	0.45	4.43	0.22	1.73	0.12	1.64	0.30	2.30
E8	0.09	1.19	0.10	0.00	0.15	3.06	0.03	0.42	0.11	2.68
E9	0.15	2.27	0.20	0.82	0.09	2.03	0.00	0.22	0.15	2.16
E10	0.12	0.00	0.07	1.79	0.03	0.29	0.00	0.25	0.00	0.49
E11	0.00	0.30	0.00	0.80	0.15	0.61	0.14	0.86	0.08	1.42
E12	0.09	1.76	0.12	0.75	0.04	1.34	17.59	37.21	0.19	4.68
E14	0.00	0.00	0.00	1.37	0.00	4.17	0.03	0.73	0.09	3.13
E15	0.86	6.25	0.39	5.88	0.71	10.62	0.65	2.74	0.69	11.17
E16	0.11	0.39	0.09	0.38	0.04	0.00	0.03	0.21	0.07	1.14
E17	0.15	0.58	0.04	0.39	0.09	0.00	0.28	0.65	0.07	0.56
E18	0.18	0.16	0.16	0.00	0.03	0.27	0.10	0.85	0.24	1.06
E20	0.03	0.00	0.00	0.00	0.09	0.00	0.25	0.36	0.09	0.11

Total CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	68.02	62.00	62.62	55.44	60.70	59.85	65.02	71.12	67.51	70.44
E2	25.39	59.00	22.85	56.46	19.19	45.06	31.99	77.86	26.26	57.90
E3	38.22	73.30	32.99	57.95	34.05	60.62	25.45	63.33	45.54	81.14
E4	37.69	65.25	36.42	63.70	34.71	66.24	33.31	75.25	37.22	63.83
E5	44.84	80.74	40.35	63.00	43.36	65.36	44.24	85.31	38.34	77.56
E6	61.82	77.88	62.38	78.72	59.82	75.00	70.46	90.83	62.73	85.31
E7	63.40	48.24	66.07	62.02	63.82	61.08	69.67	81.80	64.26	67.52
E8	68.53	68.95	65.00	60.00	65.75	65.09	71.28	80.04	62.34	65.57
E9	22.14	50.49	22.08	51.00	24.73	60.47	10.53	66.11	23.54	69.01
E10	32.24	54.17	26.71	56.16	29.66	61.73	21.79	68.49	27.60	60.09
E11	35.84	49.33	37.49	47.30	34.15	68.00	38.29	50.92	42.55	60.22
E12	36.56	57.98	37.32	63.91	31.75	61.27	41.02	65.65	43.00	73.29
E14	28.10	42.35	21.74	41.73	31.84	63.09	32.73	71.87	27.43	65.19
E15	72.44	78.45	71.70	70.83	67.21	75.81	63.31	69.12	67.55	74.27
E16	46.78	14.66	52.97	17.43	52.04	27.53	49.98	73.71	45.13	31.13
E17	68.59	73.44	68.47	72.17	68.11	74.46	75.66	85.16	66.86	64.32
E18	30.36	10.18	32.97	11.30	27.04	8.83	20.68	22.33	24.25	18.07
E20	37.39	21.51	42.63	32.34	49.48	35.84	47.09	55.72	63.13	62.05

Table A4.13b (continued):

CD45RA/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	33.80	29.56	30.69	28.47	28.14	13.43	24.41	32.75	28.48	11.17
E2	11.95	26.62	10.08	29.93	8.71	13.58	4.94	22.08	11.93	17.54
E3	18.04	40.91	14.65	27.84	14.42	21.56	6.93	29.36	16.78	9.93
E4	21.28	32.09	19.11	24.84	17.77	22.29	14.93	35.43	19.73	18.72
E5	26.43	54.81	22.40	34.00	25.47	37.07	23.21	42.89	21.13	42.44
E6	35.55	48.72	39.73	54.61	35.73	40.96	39.66	60.42	33.17	17.51
E7	32.53	34.50	32.75	38.97	29.96	22.72	20.69	26.10	31.06	18.81
E8	34.24	40.99	34.43	37.36	29.60	24.97	15.84	22.12	31.39	14.35
E9	11.53	25.49	10.41	28.51	11.26	18.88	5.12	29.86	10.42	14.62
E10	17.09	30.09	13.93	33.99	14.25	9.11	8.01	28.92	13.48	19.75
E11	15.01	26.48	18.00	25.16	8.77	17.31	15.89	25.62	19.30	16.79
E12	6.37	12.18	8.92	19.54	3.85	15.19	7.99	11.60	7.34	5.26
E14	17.36	29.41	13.57	23.62	19.30	13.73	11.76	27.20	15.50	13.27
E15	35.03	36.77	34.12	29.43	29.15	18.27	11.62	23.61	26.97	10.50
E16	23.94	9.54	27.17	9.91	25.57	14.21	12.82	27.31	25.99	18.05
E17	30.35	37.67	30.07	39.39	29.84	36.78	23.58	24.49	30.78	34.47
E18	6.69	2.96	7.14	5.01	5.46	3.15	5.54	9.77	4.46	8.96
E20	11.80	11.00	12.83	16.85	15.66	7.96	7.41	12.85	11.19	4.66

CD45RO/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	29.86	37.62	28.90	35.89	28.54	49.10	34.43	44.86	34.69	62.06
E2	10.37	41.73	9.49	29.93	7.39	30.25	18.31	54.91	10.93	43.86
E3	14.13	53.41	12.12	37.50	13.77	47.50	13.62	44.74	23.57	77.53
E4	11.73	44.39	12.25	50.32	11.82	52.23	14.44	46.68	13.04	50.64
E5	12.53	41.48	11.85	35.00	13.58	39.51	15.90	51.52	12.46	47.80
E6	17.09	46.47	16.71	40.78	16.32	46.69	19.31	30.10	23.66	74.51
E7	18.95	22.04	22.43	30.68	24.13	44.69	34.32	57.18	24.76	55.75
E8	19.85	38.72	17.32	28.79	18.39	40.12	30.33	46.33	18.47	49.52
E9	7.39	30.39	8.42	26.10	9.75	46.61	3.58	39.81	10.64	57.31
E10	10.00	32.87	8.25	31.53	10.67	51.48	8.39	32.81	8.93	38.24
E11	13.11	27.81	16.96	29.78	21.93	55.62	19.06	31.35	20.18	48.83
E12	27.16	51.26	25.92	55.63	26.69	54.79	29.44	59.96	32.20	69.59
E14	6.32	16.47	4.64	18.90	8.43	52.79	12.07	35.36	8.49	55.75
E15	30.23	61.12	31.48	57.11	31.39	69.14	36.27	49.56	33.10	70.01
E16	9.37	4.77	9.37	8.62	12.87	20.96	31.56	58.87	13.34	23.01
E17	13.53	30.62	13.08	26.18	13.69	34.95	26.48	45.23	15.27	36.41
E18	11.58	6.40	14.08	8.09	10.83	6.31	14.75	20.99	11.63	16.81
E20	14.96	13.20	18.73	17.12	17.07	28.81	28.72	39.18	26.82	52.05

TcRαβ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	1.51	12.82	0.81	10.58	0.48	7.92	1.20	9.75	0.31	7.22
E2	0.38	7.29	0.15	2.46	0.03	1.91	0.55	10.42	0.27	1.85
E3	0.21	7.14	0.39	8.33	0.16	6.39	0.55	12.92	3.10	19.79
E4	0.23	6.98	0.24	5.45	0.36	16.91	1.62	15.41	0.44	11.30
E5	0.12	11.25	0.09	6.38	0.17	7.11	4.12	33.03	1.25	11.63
E6	0.07	4.52	0.18	3.48	0.05	3.17	5.99	29.62	0.73	13.96
E7	0.05	0.79	0.05	0.00	0.07	2.33	0.79	10.59	0.05	2.81
E8	0.06	1.35	0.04	0.50	0.15	2.61	0.99	5.28	0.05	1.09
E9	0.09	0.42	0.06	1.57	0.09	8.72	0.07	6.83	0.13	4.92
E10	0.12	3.17	0.06	0.00	0.23	5.49	0.49	9.68	0.20	7.73
E11	0.11	5.48	0.17	3.05	1.11	9.92	0.40	6.85	0.89	6.91
E12	0.32	4.10	0.67	5.77	0.34	5.44	0.34	9.61	0.26	3.23
E14	0.00	2.06	0.00	1.14	0.06	3.85	0.15	2.88	0.16	5.84
E15	0.02	3.00	0.07	1.85	0.05	5.43	0.43	5.36	0.05	1.01
E16	1.59	4.83	1.65	4.84	2.07	13.16	26.94	52.22	3.84	17.25
E17	2.46	21.07	1.25	25.00	1.83	18.60	11.57	32.03	1.95	17.23
E18	0.79	1.91	1.35	2.20	1.43	3.75	8.18	20.54	3.95	13.73
E20	3.54	5.19	3.41	4.93	4.05	25.89	9.81	24.23	15.75	39.28

Table A4.13b (continued):

TcRαβ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.80	9.05	0.43	8.06	0.31	6.22	0.89	7.24	0.23	6.46
E2	0.16	6.25	0.06	2.46	0.00	1.91	0.38	8.55	0.12	1.85
E3	0.11	6.55	0.21	6.55	0.00	6.39	0.39	10.51	1.62	19.58
E4	0.09	6.98	0.06	4.46	0.18	16.91	0.52	11.65	0.18	11.74
E5	0.06	10.00	0.03	4.26	0.09	6.60	1.79	23.45	0.51	8.14
E6	0.02	3.62	0.02	3.04	0.05	2.82	0.84	9.72	0.48	13.30
E7	0.03	0.79	0.05	0.00	0.02	2.00	0.56	9.17	0.02	2.81
E8	0.00	1.08	0.02	0.00	0.00	2.43	0.54	3.70	0.00	0.92
E9	0.09	0.42	0.03	0.52	0.03	8.41	0.04	5.22	0.09	4.92
E10	0.06	3.17	0.03	0.00	0.17	5.49	0.19	5.95	0.03	6.28
E11	0.05	5.10	0.17	2.41	0.80	9.88	0.16	6.44	0.43	7.29
E12	0.29	4.92	0.41	5.77	0.34	5.99	0.34	10.59	0.33	3.85
E14	0.00	1.03	0.00	1.14	0.06	3.42	0.06	1.92	0.03	5.52
E15	0.00	4.33	0.02	2.46	0.05	6.05	0.33	4.65	0.00	1.46
E16	0.73	3.90	0.64	3.91	1.20	13.16	4.64	11.30	1.97	16.67
E17	0.61	11.95	0.00	0.00	0.30	7.85	3.56	14.16	0.52	11.82
E18	0.38	1.50	0.80	1.96	0.87	3.75	6.49	21.67	2.59	13.44
E20	1.52	5.74	1.39	3.62	1.74	24.51	4.20	10.00	7.08	31.17

TcRγδ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	3.28	8.71	2.45	8.54	3.03	7.93	1.50	2.21	2.58	5.20
E2	0.59	1.49	0.46	3.00	0.24	5.67	0.24	1.23	0.43	7.18
E3	0.52	4.00	0.81	4.64	0.61	3.46	0.13	0.81	0.57	1.21
E4	1.07	5.16	1.30	6.10	1.07	6.43	0.68	0.95	0.91	4.61
E5	1.43	1.77	0.95	3.67	1.85	6.94	0.50	1.26	1.30	5.75
E6	6.41	10.08	7.49	15.71	8.13	12.63	5.97	2.35	7.34	7.67
E7	3.37	4.42	3.26	4.87	2.82	2.67	2.76	2.65	3.31	3.96
E8	2.20	7.69	2.51	4.82	3.19	11.33	2.10	2.41	2.82	13.72
E9	1.97	6.77	1.81	4.32	2.22	7.22	0.29	0.68	2.00	4.56
E10	0.18	1.11	0.06	1.99	0.10	2.44	0.19	2.04	0.38	3.35
E11	2.35	3.51	2.50	2.79	1.12	2.83	2.53	3.81	3.22	6.46
E12	1.32	3.52	1.04	4.47	0.88	2.03	1.27	3.23	1.50	1.11
E14	0.29	0.00	0.23	1.63	0.52	3.33	0.16	0.93	0.52	2.10
E15	0.61	3.52	1.13	19.71	0.51	1.55	0.72	0.98	0.87	1.22
E16	2.43	4.17	1.78	3.19	1.53	9.11	3.42	8.40	4.13	14.24
E17	5.19	7.19	6.17	9.34	ND	ND	4.45	7.81	6.57	17.49
E18	6.35	2.23	5.27	2.59	6.12	4.93	10.47	16.29	6.50	12.52
E20	3.26	6.02	0.98	2.14	0.67	7.14	4.20	15.78	0.47	2.36

TcRγδ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.98	5.71	0.67	4.69	0.81	4.34	0.56	1.46	0.81	3.26
E2	0.08	0.75	0.06	2.00	0.06	4.96	0.07	1.04	0.00	6.63
E3	0.26	3.43	0.29	3.97	0.21	2.60	0.07	0.35	0.24	1.21
E4	0.18	4.52	0.18	4.88	0.24	6.43	0.16	0.81	0.12	3.69
E5	0.06	1.77	0.06	2.75	0.06	3.47	0.07	0.59	0.06	3.45
E6	0.21	3.23	0.92	10.00	0.90	8.07	0.64	0.52	0.59	3.31
E7	0.59	3.54	0.38	3.33	0.38	2.00	0.60	1.91	0.43	2.90
E8	0.50	4.90	0.25	2.63	0.57	7.81	0.40	1.13	0.42	9.06
E9	0.26	4.17	0.12	2.16	0.24	4.64	0.04	0.00	0.42	2.13
E10	0.09	1.11	0.00	1.32	0.00	1.63	0.11	1.25	0.19	2.87
E11	0.31	1.83	0.38	1.47	0.54	1.49	0.33	1.97	0.84	4.05
E12	0.45	3.52	0.37	4.12	0.85	1.82	0.58	3.46	0.61	1.11
E14	0.00	0.00	0.00	1.63	0.15	2.96	0.06	0.72	0.13	2.10
E15	0.25	3.27	0.53	20.67	0.26	0.42	0.37	0.93	0.61	0.71
E16	1.12	4.55	0.50	3.19	0.35	8.43	1.15	3.78	2.29	15.63
E17	0.25	2.74	0.34	3.81	ND	ND	1.25	4.09	0.58	12.02
E18	1.12	1.96	0.66	1.77	1.43	4.57	6.73	20.17	2.60	13.28
E20	1.42	5.73	0.43	1.83	0.52	7.83	2.42	9.16	0.00	1.11

Table A4.13b (continued):

CD4/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	40.59	50.00	49.82	52.27	44.79	55.79	23.01	35.90	43.28	60.18
E2	19.66	45.74	19.11	45.19	17.78	35.29	7.69	29.95	19.39	47.83
E3	28.59	54.33	29.49	51.61	27.41	51.06	14.63	43.72	36.15	77.79
E4	27.75	51.43	26.32	50.29	23.06	41.82	23.07	53.02	25.67	54.04
E5	25.09	56.62	24.69	48.28	28.44	54.59	27.87	61.17	29.52	50.29
E6	30.61	45.00	27.70	41.51	28.67	45.49	22.41	31.12	32.07	72.42
E7	41.37	40.94	43.56	40.39	44.13	50.71	35.29	49.33	39.78	57.50
E8	43.17	48.37	40.47	38.52	42.34	45.35	20.67	28.30	38.53	42.69
E9	13.31	31.22	13.86	35.26	12.80	42.97	3.41	17.98	13.18	54.74
E10	20.05	30.20	18.40	39.16	21.58	58.76	9.07	27.08	15.42	42.23
E11	24.52	37.85	24.36	33.52	9.88	19.81	24.37	37.35	24.14	47.56
E12	31.50	52.75	31.54	52.85	17.56	38.41	32.99	53.33	35.05	65.47
E14	15.80	33.67	15.12	27.43	16.70	52.25	14.80	32.84	18.45	52.66
E15	47.59	54.98	51.47	61.09	47.17	67.19	19.13	23.37	47.81	66.50
E16	31.29	14.99	38.65	23.70	34.58	29.94	23.66	38.16	31.83	27.16
E17	46.46	52.63	43.32	60.77	45.75	54.42	41.97	52.33	48.12	56.42
E18	18.44	7.37	16.68	5.56	15.14	5.57	11.70	19.42	13.80	15.15
E20	25.64	19.75	26.40	18.54	28.28	35.41	25.91	29.13	47.43	56.19

CD4/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	21.64	36.92	28.06	43.56	26.58	51.99	14.25	22.66	28.16	57.83
E2	7.61	27.13	7.61	24.04	7.38	28.57	4.77	20.82	7.12	35.40
E3	9.76	37.98	9.93	32.26	11.15	44.26	8.89	32.26	16.80	71.93
E4	9.53	38.57	8.38	38.73	8.57	32.12	8.73	28.02	9.22	47.66
E5	8.31	25.00	9.67	21.55	10.60	34.78	10.86	31.19	12.36	33.14
E6	9.97	33.46	8.92	28.30	11.22	36.10	7.77	12.63	15.09	65.11
E7	18.98	23.94	19.79	26.52	21.29	36.71	20.09	33.43	22.01	48.76
E8	15.11	29.63	14.27	20.42	16.10	30.18	9.16	16.70	15.24	36.12
E9	6.17	18.99	6.99	23.12	6.68	35.54	0.88	8.26	7.45	46.61
E10	6.44	15.35	6.18	24.48	9.49	48.20	4.10	14.29	6.19	25.50
E11	15.54	29.31	16.18	25.76	7.61	17.41	16.25	32.03	17.28	44.73
E12	25.40	45.80	25.02	49.85	15.91	36.21	26.93	51.19	28.48	64.90
E14	4.12	22.45	3.39	15.93	7.12	47.40	8.45	25.80	8.26	47.85
E15	25.87	48.42	26.73	56.27	24.36	65.36	11.83	17.57	27.08	64.49
E16	7.48	6.45	12.62	16.82	12.06	25.69	8.92	18.48	11.19	23.07
E17	16.81	24.34	15.89	31.56	14.59	27.56	18.26	32.57	18.09	36.31
E18	11.80	5.36	10.81	4.66	9.89	5.45	9.75	19.75	8.79	14.29
E20	15.64	13.75	16.15	14.33	16.33	29.73	18.26	18.57	31.17	46.62

CD8/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	22.57	35.35	21.21	29.60	19.74	20.08	1.32	1.51	18.81	12.12
E2	6.70	21.11	4.73	15.93	4.32	18.18	15.53	62.39	4.60	16.05
E3	5.76	19.13	4.37	27.27	5.26	15.30	6.69	28.33	4.92	7.25
E4	10.55	40.62	8.30	33.73	8.57	37.70	11.30	36.94	9.62	27.07
E5	11.70	46.09	9.16	39.82	12.08	35.95	14.00	32.78	12.21	30.72
E6	27.75	51.04	26.21	43.32	26.21	45.25	44.23	71.47	24.83	20.55
E7	17.83	24.88	18.76	26.71	17.27	19.27	31.56	47.78	16.84	15.18
E8	18.45	33.89	18.00	32.28	19.30	25.17	49.55	66.95	17.38	16.43
E9	6.94	33.97	7.73	18.52	7.92	17.30	6.21	57.58	8.36	15.65
E10	7.97	25.55	7.81	36.42	8.92	14.29	11.01	41.71	5.85	21.24
E11	9.33	26.89	9.31	21.98	22.39	59.40	9.95	20.49	11.74	17.55
E12	5.80	21.11	5.11	20.87	11.91	34.63	5.58	17.71	5.21	5.85
E14	5.06	17.86	5.33	20.56	5.06	16.49	16.01	47.85	6.01	11.99
E15	17.68	52.67	18.13	52.40	14.72	25.17	39.89	58.08	15.59	16.57
E16	12.08	7.83	13.80	9.95	13.39	16.16	27.37	54.24	13.75	20.80
E17	17.85	30.41	20.00	26.12	18.17	30.33	35.47	48.75	19.41	25.80
E18	8.48	5.60	6.61	3.60	7.46	5.00	13.92	18.59	11.47	13.97
E20	13.54	14.13	13.52	10.82	17.50	25.63	28.50	48.57	15.48	9.33

Table A4.13b (continued):

CD8/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	2.34	16.28	1.69	10.20	2.06	11.94	0.75	0.90	1.74	7.63
E2	0.27	5.00	0.55	7.08	0.28	5.45	2.53	15.18	0.30	6.17
E3	0.32	6.09	0.18	8.52	0.24	4.10	0.58	8.33	0.27	4.39
E4	1.19	12.50	0.66	14.20	0.76	14.75	1.34	9.12	0.87	14.41
E5	0.70	13.04	0.56	7.96	0.80	15.03	1.13	8.46	0.67	9.04
E6	3.75	23.96	2.35	16.61	3.10	22.62	4.36	7.41	2.33	12.40
E7	1.11	5.91	0.78	7.05	1.07	8.12	2.90	10.51	1.01	6.90
E8	1.07	5.97	1.12	6.93	0.74	8.33	3.29	7.50	0.89	8.25
E9	0.21	10.53	0.54	5.29	0.44	5.57	0.26	7.58	0.53	7.71
E10	0.49	5.84	0.35	9.88	0.77	5.17	1.10	6.55	0.35	7.25
E11	0.98	6.03	0.49	6.61	3.94	16.36	0.51	6.02	0.90	8.82
E12	1.09	9.68	1.18	8.74	2.63	11.04	1.44	11.23	1.29	3.75
E14	0.10	2.38	0.07	7.48	0.09	6.45	0.61	5.98	0.20	6.94
E15	2.71	31.28	2.68	28.37	2.70	14.55	3.98	10.89	3.06	12.09
E16	2.45	5.57	2.73	8.14	2.84	13.64	6.56	16.19	4.16	18.61
E17	2.75	14.86	3.44	13.20	3.33	20.33	13.93	26.58	2.63	15.69
E18	3.01	4.31	3.07	2.98	3.31	4.74	8.52	18.59	7.68	14.71
E20	1.79	5.87	1.76	3.51	1.78	20.85	13.62	22.90	0.97	5.18

TcR Vγ9/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	2.34	7.31	2.66	5.83	5.36	9.77	38.60	56.37	2.31	2.45
E2	0.85	2.31	0.34	0.00	0.35	2.06	0.25	0.61	0.42	0.50
E3	0.63	4.15	0.64	1.74	0.62	1.40	0.29	0.47	0.49	0.68
E4	0.64	5.93	0.83	3.29	0.85	4.03	0.75	1.64	0.66	0.83
E5	0.74	0.90	0.63	6.03	0.69	2.76	0.32	0.33	0.69	3.27
E6	1.89	1.97	1.47	3.24	2.35	2.17	1.32	0.81	1.82	1.98
E7	1.78	3.54	1.80	2.36	2.02	1.61	1.30	1.00	2.05	0.66
E8	1.79	5.28	1.58	2.12	1.85	2.31	1.05	0.91	1.68	2.29
E9	0.68	5.19	0.82	4.81	0.88	3.91	0.11	0.47	0.71	2.76
E10	0.06	2.34	0.25	3.79	0.17	0.45	0.11	0.47	0.30	1.63
E11	1.53	2.04	1.66	2.73	0.64	0.45	1.67	0.90	1.81	1.40
E12	0.94	3.62	1.16	3.78	0.89	1.90	1.66	3.08	1.62	1.08
E14	0.29	5.26	0.16	1.92	0.25	1.91	0.47	0.67	0.42	2.62
E15	0.57	3.24	0.78	1.72	0.58	0.63	0.54	0.45	0.59	0.65
E16	0.64	2.09	1.08	4.81	0.76	3.88	1.29	4.05	1.47	10.38
E17	3.42	6.16	3.06	5.40	3.41	8.43	2.71	4.45	3.64	7.14
E18	2.42	2.31	2.92	1.66	2.53	2.94	7.20	11.96	4.85	13.83
E20	1.39	4.41	1.15	2.34	0.60	13.16	1.28	7.70	0.27	4.93

TcR Vγ9/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.50	3.16	0.51	2.80	1.61	6.14	5.51	10.68	0.47	1.43
E2	0.06	1.16	0.00	0.00	0.03	0.00	0.18	0.46	0.00	0.50
E3	0.05	2.59	0.08	1.16	0.13	0.70	0.13	0.00	0.03	0.29
E4	0.24	5.93	0.00	1.97	0.12	2.01	0.26	0.60	0.06	0.83
E5	0.03	0.90	0.00	0.86	0.03	1.66	0.03	0.25	0.00	0.65
E6	0.48	1.57	0.23	1.18	0.58	1.09	0.47	0.53	0.27	1.40
E7	0.34	1.36	0.19	1.42	0.22	1.25	0.47	0.74	0.17	0.40
E8	0.10	2.94	0.04	1.06	0.21	0.43	0.26	0.40	0.07	1.46
E9	0.06	1.89	0.21	2.67	0.47	2.51	0.07	0.24	0.06	0.92
E10	0.03	0.58	0.06	0.76	0.03	0.22	0.07	0.16	0.00	1.22
E11	0.08	0.14	0.29	1.50	0.24	0.27	0.14	0.39	0.23	0.61
E12	0.39	1.97	0.18	2.77	0.50	1.61	0.38	2.61	0.29	0.97
E14	0.06	1.32	0.07	0.96	0.03	1.91	0.34	0.56	0.10	2.33
E15	0.32	2.65	0.25	1.15	0.31	0.63	0.29	0.23	0.11	0.65
E16	0.15	1.33	0.55	4.81	0.28	4.07	0.46	2.16	0.76	10.18
E17	0.50	3.77	0.29	1.42	0.53	3.93	1.39	2.71	0.43	4.86
E18	0.75	1.73	0.90	1.40	0.73	2.94	5.91	13.83	2.94	14.09
E20	0.64	4.68	0.79	4.35	0.44	12.94	0.94	4.74	0.12	3.32

Table A4.13b (continued):

TcR Vδ1/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	1.36	4.32	1.32	4.74	1.50	2.65	1.48	1.98	1.61	3.77
E2	0.23	1.06	0.38	2.29	0.20	1.40	0.27	1.65	0.23	0.40
E3	0.03	0.56	0.00	0.64	0.00	0.33	0.03	0.13	0.03	0.10
E4	1.05	2.92	0.98	2.60	0.95	0.64	0.88	1.57	0.91	4.57
E5	1.70	0.90	1.39	1.06	1.55	1.84	1.04	0.54	2.23	2.63
E6	5.93	7.05	6.22	8.55	6.83	8.14	5.51	2.08	6.88	7.60
E7	1.62	0.48	1.86	1.51	1.78	1.41	2.22	1.63	2.17	2.22
E8	2.10	2.55	2.14	1.65	2.82	6.27	3.29	2.13	3.65	16.51
E9	1.35	1.42	1.77	2.48	2.45	5.75	0.37	2.87	2.07	2.18
E10	0.12	1.18	0.12	0.00	0.10	0.50	0.11	0.45	0.13	0.44
E11	2.03	4.80	1.75	3.10	1.98	3.89	1.67	6.05	2.67	6.36
E12	0.33	1.03	0.34	0.59	0.25	0.58	0.36	1.30	0.38	0.86
E14	0.06	0.00	0.13	1.25	0.12	1.29	0.30	0.50	0.10	0.37
E15	0.28	0.29	0.45	1.62	0.36	1.36	0.86	0.97	0.41	0.42
E16	0.85	1.83	0.98	2.48	0.86	6.68	2.82	8.67	2.45	14.63
E17	2.01	5.15	2.14	1.33	1.68	3.79	1.85	4.24	1.76	6.43
E18	3.39	1.33	3.30	1.62	3.27	3.07	9.51	23.27	6.08	16.23
E20	1.76	4.30	1.08	2.90	1.12	15.53	1.70	8.00	0.56	2.39

TcR Vδ1/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	0.09	1.69	0.06	0.95	0.10	1.42	0.10	0.99	0.31	1.83
E2	0.00	1.06	0.03	0.00	0.00	0.70	0.17	1.10	0.00	0.00
E3	0.00	0.56	0.00	0.64	0.00	0.33	0.03	0.13	0.00	0.10
E4	0.03	0.73	0.12	1.30	0.00	0.64	0.00	0.48	0.00	2.28
E5	0.03	0.90	0.00	0.00	0.00	0.61	0.00	0.15	0.03	0.66
E6	0.06	3.52	0.02	3.95	0.14	3.88	0.05	0.45	0.13	2.71
E7	0.05	0.48	0.14	0.86	0.09	0.16	0.20	0.90	0.02	0.89
E8	0.02	0.77	0.04	0.62	0.06	2.20	0.24	0.60	0.50	10.18
E9	0.03	0.47	0.00	0.00	0.03	2.88	0.00	0.41	0.03	0.48
E10	0.03	0.00	0.00	0.00	0.07	0.50	0.00	0.00	0.00	0.00
E11	0.06	2.49	0.09	0.90	0.28	1.94	0.03	2.67	0.14	3.06
E12	0.03	1.03	0.00	0.59	0.00	0.22	0.09	0.87	0.06	0.65
E14	0.00	0.00	0.06	1.25	0.03	0.32	0.03	0.20	0.00	0.00
E15	0.02	0.00	0.02	1.62	0.00	0.61	0.21	0.64	0.11	0.53
E16	0.21	1.43	0.18	2.07	0.17	5.85	0.69	4.32	1.54	15.51
E17	0.08	1.82	0.07	0.33	0.08	1.03	0.16	1.19	0.06	4.09
E18	0.78	0.59	0.56	1.08	0.86	2.69	6.90	26.89	3.57	15.88
E20	0.99	5.02	0.62	2.58	0.82	16.84	0.76	4.17	0.09	1.39

Table A4.14: Complete data for total numbers of cells (per 10^6 cells plated on day 0) of cellular subsets for each exposed individual on a) day 0 and b) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)
cells in gate

DONOR	R1	R2
E1	356400	389100
E2	90800	102300
E3	342700	46200
E4	266100	20300
E5	276500	15400
E6	529600	52800
E7	556000	75000
E8	477500	63800
E9	389400	114200
E10	225800	59300
E11	438300	191000
E12	467300	31900
E14	485400	17900
E15	708800	47800
E16	657700	48000
E17	646400	52500
E18	621500	50700
E20	508100	22900

CD20

DONOR	R1	R2
E1	54280	32334
E2	3959	17790
E3	31768	3229
E4	20889	2436
E5	32433	2304
E6	26851	6236
E7	66053	7290
E8	6494	4887
E9	38395	13099
E10	25967	7413
E11	108348	76400
E12	49814	4530
E14	65092	3491
E15	61666	8069
E16	57878	3763
E17	44860	23147
E18	92479	17517
E20	23423	2498

CD20/CD45RO

DONOR	R1	R2
E1	606	1167
E2	0	184
E3	2330	448
E4	0	0
E5	0	60
E6	0	0
E7	389	353
E8	334	223
E9	0	388
E10	0	0
E11	131	0
E12	0	309
E14	97	149
E15	496	86
E16	197	82
E17	129	567
E18	124	461
E20	203	277

Table A4.14a (continued):

CD56

DONOR	R1	R2
E1	2031	7082
E2	0	2997
E3	27005	7355
E4	985	181
E5	1051	302
E6	1271	417
E7	2057	1995
E8	13179	8587
E9	1558	3666
E10	858	1666
E11	438	688
E12	1215	412
E14	777	446
E15	1772	607
E16	3486	734
E17	2586	452
E18	1554	304
E20	864	417

CD56/CD45RO

DONOR	R1	R2
E1	143	467
E2	0	0
E3	25086	6639
E4	106	0
E5	0	60
E6	0	0
E7	111	233
E8	1051	740
E9	0	194
E10	0	184
E11	219	344
E12	187	102
E14	97	149
E15	213	172
E16	66	82
E17	323	226
E18	186	152
E20	0	140

Total CD3

DONOR	R1	R2
E1	263879	299529
E2	44846	65636
E3	221761	27323
E4	167270	10718
E5	147015	10281
E6	327293	32414
E7	360622	44400
E8	303260	25788
E9	183407	73191
E10	126222	35544
E11	243257	86122
E12	278885	15720
E14	241584	7511
E15	421169	21214
E16	398303	15226
E17	478724	17766
E18	391421	20229
E20	144961	12437

Table A4.14a (continued):

CD45RA/CD3

DONOR	R1	R2
E1	160594	155056
E2	23045	19918
E3	92460	8445
E4	90900	5034
E5	92213	5208
E6	227675	18343
E7	201272	19208
E8	173285	11541
E9	97934	42380
E10	67830	14505
E11	127764	42727
E12	73693	4252
E14	155765	3897
E15	201228	8580
E16	234141	6240
E17	292690	6662
E18	148539	10115
E20	102738	8489

CD45RO/CD3

DONOR	R1	R2
E1	95800	158831
E2	17833	45268
E3	109253	18628
E4	60777	6171
E5	41364	5435
E6	73879	17371
E7	122876	23550
E8	95070	12377
E9	61915	44766
E10	50760	24663
E11	85688	51150
E12	166733	12945
E14	56695	3501
E15	192864	16309
E16	101088	7507
E17	111698	8437
E18	168427	14414
E20	22712	4443

TcRαβ/CD3

DONOR	R1	R2
E1	7556	24435
E2	445	1514
E3	3461	1538
E4	5508	1052
E5	9539	901
E6	25580	5027
E7	16458	2738
E8	24257	4351
E9	14330	8896
E10	1535	166
E11	131	630
E12	93	542
E14	0	70
E15	921	492
E16	66	91
E17	776	809
E18	373	380
E20	102	266

Table A4.14a (continued):

TcRαβ/CD45RO

DONOR	R1	R2
E1	1140	5292
E2	163	501
E3	1337	1026
E4	213	323
E5	442	225
E6	4078	1410
E7	2391	495
E8	1194	664
E9	2414	3632
E10	565	166
E11	0	1280
E12	0	453
E14	0	70
E15	638	368
E16	0	91
E17	129	693
E18	0	289
E20	102	266

TcRγδ/CD3

DONOR	R1	R2
E1	12082	29572
E2	781	7488
E3	3153	1599
E4	6839	497
E5	5143	453
E6	20654	6204
E7	13622	5378
E8	23016	4766
E9	11293	9353
E10	2168	1411
E11	30725	14096
E12	14346	1557
E14	5922	199
E15	12617	1525
E16	12628	1262
E17	27989	3014
E18	72653	2956
E20	11534	2306

TcRγδ/CD45RO

DONOR	R1	R2
E1	3029	6809
E2	281	4992
E3	1268	725
E4	639	213
E5	415	226
E6	2701	3152
E7	2057	1590
E8	1671	612
E9	584	5379
E10	745	1257
E11	2016	5004
E12	4019	983
E14	534	0
E15	2623	970
E16	1250	360
E17	2715	1565
E18	808	228
E20	203	769

Table A4.14a (continued):

CD4/CD3

DONOR	R1	R2
E1	157279	177391
E2	27721	54863
E3	163948	23100
E4	107904	7375
E5	97383	6160
E6	122020	15360
E7	14400	4178
E8	170802	13424
E9	84149	35733
E10	80294	25297
E11	110320	53308
E12	169209	13573
E14	135038	6150
E15	219303	21481
E16	246374	21850
E17	307169	16328
E18	193659	21664
E20	79975	7669

CD4/CD45RO

DONOR	R1	R2
E1	55563	93267
E2	7800	26864
E3	63468	14345
E4	30468	4600
E5	26212	3031
E6	25633	7487
E7	2113	1433
E8	41686	6029
E9	44820	27522
E10	34254	15756
E11	50492	18164
E12	108414	10278
E14	28153	2280
E15	87891	16219
E16	52156	6917
E17	61149	6059
E18	91920	12604
E20	13160	2663

CD8/CD3

DONOR	R1	R2
E1	70567	123345
E2	11895	42127
E3	37526	15149
E4	39250	5483
E5	42775	4760
E6	109839	22387
E7	221622	26460
E8	87048	12371
E9	57787	40358
E10	29489	17072
E11	67148	55753
E12	33646	6945
E14	47084	6165
E15	114329	10741
E16	104903	11515
E17	103876	12248
E18	76942	8954
E20	37549	5285

Table A4.14a (continued):

CD8/CD45RO

DONOR	R1	R2
E1	4170	13969
E2	1226	4511
E3	1234	2019
E4	2129	1186
E5	1355	414
E6	6673	3173
E7	88015	16838
E8	4250	957
E9	4400	9250
E10	3635	3819
E11	2323	2903
E12	6028	1372
E14	1747	132
E15	24879	2032
E16	3946	509
E17	5947	1460
E18	14543	2216
E20	203	414

TcR Vγ9/CD3

DONOR	R1	R2
E1	10763	25525
E2	817	931
E3	30603	6440
E4	5428	546
E5	2571	533
E6	11069	2297
E7	102971	19148
E8	11365	1359
E9	8216	5310
E10	1626	670
E11	24107	5673
E12	16683	947
E14	3689	138
E15	6167	502
E16	9010	878
E17	30445	3166
E18	39776	1080
E20	1829	261

TcR Vγ9/CD45RO

DONOR	R1	R2
E1	392	6809
E2	82	0
E3	5963	1959
E4	293	274
E5	111	106
E6	1271	1357
E7	4837	1830
E8	334	325
E9	1480	2090
E10	542	445
E11	657	1776
E12	748	118
E14	0	68
E15	1488	201
E16	263	82
E17	388	1586
E18	808	542
E20	0	0

Table A4.14a (continued):

TcR Vδ1/CD3

DONOR	R1	R2
E1	8767	13190
E2	708	696
E3	411	961
E4	5934	296
E5	7355	1067
E6	27486	2936
E7	10453	3413
E8	36863	5174
E9	13512	7788
E10	881	0
E11	29980	14019
E12	2290	338
E14	3495	200
E15	3828	593
E16	7761	744
E17	10472	1418
E18	53946	2834
E20	15040	2487

TcR Vδ1/CD45RO

DONOR	R1	R2
E1	214	584
E2	45	0
E3	206	0
E4	0	148
E5	0	0
E6	0	269
E7	723	570
E8	812	338
E9	117	1142
E10	0	0
E11	131	1070
E12	93	450
E14	291	100
E15	284	167
E16	0	278
E17	323	982
E18	497	1486
E20	0	524

Table A4.14b:

b)
cells in gate

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	92993	10913	140850	12750	125782	22082	154148	96730	115847	32509
E2	71720	8338	69321	5208	66880	4940	96866	66970	74140	6490
E3	188279	34131	167200	4180	163152	7788	122496	40934	193260	53940
E4	127300	4446	128250	3975	160332	7812	82964	23520	159173	8693
E5	108605	1972	193724	9737	98952	6076	200898	91930	163482	8054
E6	236425	9996	198870	12096	190715	11664	206416	98407	192206	32398
E7	120060	5910	168947	13869	160004	16796	126720	100692	102398	151425
E8	143988	63190	109940	10860	166010	18785	105216	86976	209950	55243
E9	181328	15568	245073	15251	142516	18876	137713	26968	152390	18032
E10	86996	4914	57114	2934	90240	15390	49580	16700	105157	9347
E11	112800	17472	100330	20827	105690	23040	45700	48460	115659	50765
E12	79850	9325	58238	7104	147004	16694	56201	37367	160217	54024
E14	97377	1669	137808	8135	125438	12675	120384	38912	64370	8364
E15	232024	9700	106128	3520	118748	16800	92628	60669	230841	56570
E16	238310	29101	300048	31725	194130	23940	324342	319791	241428	37262
E17	242606	15136	195098	12935	205641	12775	193490	103235	223744	16720
E18	137492	22940	142074	26082	136850	29070	71418	25190	90038	18460
E20	163485	13755	265210	22110	141284	28690	156676	76544	407523	237533

CD20

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	9904	1467	15479	1459	11761	2568	20363	25817	7518	3134
E2	2345	834	1227	229	2655	810	4795	14613	2973	731
E3	6646	3539	4832	425	5237	783	6321	9611	4600	4973
E4	6696	527	4591	450	5195	1098	4447	4575	5030	1159
E5	5191	176	9822	567	5492	588	12154	14194	9825	1086
E6	8724	1057	6225	895	7514	1224	8566	11248	7323	2142
E7	11394	313	15019	692	16768	1414	10340	18638	8089	13341
E8	9474	5662	8213	1467	11405	2555	5692	14908	10057	4552
E9	9121	2123	9435	1251	6142	2680	0	59	4175	1998
E10	3349	653	1691	262	3411	2461	2256	3719	3344	1421
E11	19165	5561	15401	6046	10897	5283	6951	12304	16944	8838
E12	3985	1149	2533	1011	6439	3426	12999	25204	5191	3285
E14	2834	14	3183	445	4729	1954	9053	7249	1976	691
E15	17355	1984	7280	565	6294	1614	6753	16957	7387	3994
E16	10629	1775	13712	895	8600	991	14174	48704	18638	10825
E17	14144	1488	14944	916	25972	650	18401	16466	27274	4063
E18	6930	395	9760	237	10756	1244	12927	7570	8923	3299
E20	37258	2710	36095	4559	20952	11341	29659	33725	26000	61046

CD20/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	102	216	0	524	101	428	92	2592	70	644
E2	0	111	42	46	40	122	165	2136	22	56
E3	56	416	33	177	0	92	0	1310	58	1586
E4	38	59	38	37	96	65	0	355	48	124
E5	33	18	0	189	30	118	241	2298	0	192
E6	0	81	40	149	76	0	186	502	0	87
E7	60	47	253	115	112	144	393	3232	20	2059
E8	58	569	0	117	0	351	53	1218	42	475
E9	54	0	147	0	86	274	0	0	0	146
E10	52	0	0	0	0	0	20	124	32	0
E11	90	105	90	223	127	829	14	504	0	482
E12	96	65	17	107	59	297	4535	12043	48	216
E14	29	0	0	55	0	53	36	615	0	95
E15	93	220	0	56	24	170	65	1911	0	289
E16	214	402	60	0	136	41	0	352	579	1390
E17	291	88	78	50	185	109	542	568	112	373
E18	330	71	114	34	151	78	193	426	216	508
E20	262	0	159	380	0	525	517	1102	367	808

Table A4.14b (continued):

CD56

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	3264	1617	3789	859	5522	3509	3962	2254	6429	3693
E2	868	222	665	183	776	1053	4436	5471	1461	1011
E3	7192	7075	5969	354	8060	1797	2695	4544	8851	5739
E4	3386	702	3104	600	2341	710	863	621	3852	1241
E5	2411	194	3681	945	2147	784	2893	1912	4627	1279
E6	16030	1463	13384	2539	11424	1399	7390	2647	15665	3761
E7	4166	297	7501	2112	7920	1161	3523	3192	8182	17429
E8	24262	11317	20306	2231	28703	4600	13005	2975	46609	16297
E9	4805	1178	7499	750	6755	2516	0	59	5852	1560
E10	1957	309	1097	105	3149	1431	545	331	3113	595
E11	508	236	291	527	687	346	334	1672	1029	2305
E12	1086	591	786	266	3631	1092	22390	25574	4887	7315
E14	146	0	358	223	2333	1268	506	568	1281	1072
E15	11207	1599	4755	621	9274	3342	6540	3761	20522	13362
E16	7078	1487	8641	1075	4562	1652	8692	20754	7943	6472
E17	13271	963	9657	1018	10261	1246	7740	2983	15349	2523
E18	4455	216	4191	68	6213	738	3785	3119	5429	2031
E20	1586	616	1856	683	1837	3979	3102	3253	5868	9026

CD56/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	270	151	141	149	38	598	92	764	197	731
E2	108	111	21	46	60	203	0	1112	44	140
E3	301	625	167	0	179	692	122	1400	155	1969
E4	153	59	141	112	144	65	25	207	143	289
E5	0	0	58	94	59	0	301	956	147	128
E6	1631	407	1432	1045	782	262	991	413	1249	1487
E7	204	31	760	614	352	291	152	1651	307	3483
E8	130	752	110	0	249	575	32	365	231	1480
E9	272	353	490	125	128	383	0	59	229	389
E10	104	0	40	53	27	45	0	42	0	46
E11	0	52	0	167	159	141	64	417	93	721
E12	72	164	70	53	59	224	9886	13904	304	2528
E14	0	0	0	111	0	529	36	284	58	262
E15	1995	606	414	207	843	1784	602	1662	1593	6319
E16	262	113	270	121	78	0	97	672	169	425
E17	364	88	78	50	185	0	542	671	157	94
E18	247	37	227	0	41	78	71	214	216	196
E20	49	0	0	0	127	0	392	276	367	261

Total CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	63253	6766	88200	7069	76350	13216	100227	68794	78208	22899
E2	18210	4919	15840	2940	12834	2226	30987	52143	19469	3758
E3	71960	25018	55159	2422	55553	4721	31175	25923	88011	43767
E4	47979	2901	46709	2532	55651	5175	27635	17699	59244	5548
E5	48698	1592	78167	6134	42906	3971	88877	78425	62679	6246
E6	146158	7785	124055	9522	114085	8748	145441	89383	120571	27639
E7	76118	2851	111623	8602	102115	10259	88286	82366	65801	102242
E8	98675	43570	71461	6516	109152	12227	74998	69616	130883	36223
E9	40146	7860	54112	7778	35244	11414	14501	17828	35873	12444
E10	28048	2662	15255	1648	26765	9500	10803	11438	29023	5616
E11	40428	8619	37614	9851	36093	15667	17499	24676	49213	30571
E12	29193	5407	21734	4540	46674	10228	23054	24531	68893	39594
E14	27363	707	29959	3395	39939	7997	39402	27966	17657	5452
E15	168078	7610	76094	2493	79811	12736	58642	41934	155933	42014
E16	111481	4266	158935	5530	101025	6591	162106	235718	108956	11600
E17	166403	11116	133583	9335	140062	9512	146395	87915	149595	10754
E18	41743	2335	46842	2947	37004	2567	14769	5625	21834	3336
E20	61127	2959	113059	7150	69907	10282	73779	42650	257269	147389

Table A4.14b (continued):

CD45RA/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	31431	3226	43227	3630	35395	2966	37627	31679	32993	3631
E2	8571	2220	6988	1559	5825	671	4785	14787	8845	1138
E3	33965	13963	24495	1164	23527	1679	8489	12018	32429	5356
E4	27089	1427	24509	987	28491	1741	12387	8333	31405	1627
E5	28704	1081	43394	3311	25203	2252	46628	39429	34544	3418
E6	84049	4870	79011	6606	68142	4778	81865	59457	63755	5673
E7	39056	2039	55330	5405	47937	3816	26218	26281	31805	28483
E8	49301	25902	37852	4057	49139	4691	16666	19239	65903	7927
E9	20907	3968	25512	4348	16047	3564	7051	8052	15879	2636
E10	14868	1479	7956	997	12859	1402	3971	4830	14175	1846
E11	16931	4627	18059	5240	9269	3988	7262	12415	22322	8523
E12	5086	1136	5195	1388	5660	2536	4490	4335	11760	2842
E14	16905	491	18701	1921	24209	1740	14157	10584	9977	1110
E15	81278	3567	36211	1036	34615	3069	10763	14324	62258	5940
E16	57051	2776	81523	3144	49639	3402	41581	87335	62747	6726
E17	73631	5702	58666	5095	61363	4699	45625	25282	68868	5763
E18	9198	679	10144	1307	7472	916	3957	2461	4016	1654
E20	19291	1513	34026	3726	22125	2284	11610	9836	45602	11069

CD45RO/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	27768	4105	40706	4576	35898	10842	53073	43393	40187	20175
E2	7437	3479	6579	1559	4942	1494	17736	36773	8104	2847
E3	26604	18229	20265	1568	22466	3699	16684	18314	45551	41820
E4	14932	1974	15711	2000	18951	4080	11980	10979	20756	4402
E5	13608	818	22956	3408	13438	2401	31943	47362	20370	3850
E6	40405	4645	33231	4933	31125	5446	39859	29620	45476	24140
E7	22751	1303	37895	4255	38609	7506	43490	57576	25354	84419
E8	28582	24467	19042	3127	30529	7537	31912	40296	38778	27356
E9	13400	4731	20635	3981	13895	8798	4930	10736	16214	10334
E10	8700	1615	4712	925	9629	7923	4160	5479	9390	3574
E11	14788	4859	17016	6202	23178	12815	8710	15192	23340	24789
E12	21687	4780	15095	3952	39235	9147	16546	22405	51590	37595
E14	6154	275	6394	1537	10574	6691	14530	13759	5465	4663
E15	70141	5929	33409	2010	37275	11616	33596	30067	76408	39604
E16	22330	1388	28114	2735	24985	5018	102362	188261	32206	8574
E17	32825	4635	25519	3386	28152	4465	51236	46693	34166	6088
E18	15922	1468	20004	2110	14821	1834	10534	5287	10471	3103
E20	24457	1816	49674	3785	24117	8266	44997	29990	109298	123636

TcRαβ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	1404	1399	1141	1349	604	1749	1850	9431	359	2347
E2	273	608	104	128	20	94	533	6978	200	120
E3	395	2437	652	348	261	498	674	5289	5991	10675
E4	293	310	308	217	577	1321	1344	3624	700	982
E5	130	222	174	621	168	432	8277	30364	2044	937
E6	165	452	358	421	95	370	12364	29148	1403	4523
E7	60	47	84	0	112	391	1001	10663	51	4255
E8	86	853	44	54	249	490	1042	4592	105	602
E9	163	65	147	239	128	1646	96	1842	198	887
E10	104	156	34	0	208	845	243	1617	210	722
E11	124	957	171	635	1173	2286	183	3320	1029	3508
E12	256	382	390	410	500	908	191	3591	417	1745
E14	0	34	0	93	75	488	181	1121	103	488
E15	46	291	74	65	59	912	398	3252	115	571
E16	3789	1406	4951	1535	4018	3151	87378	166995	9271	6428
E17	5968	3189	2439	3234	3763	2376	22387	33066	4363	2881
E18	1086	438	1918	574	1957	1090	5842	5174	3557	2535
E20	5787	714	9044	1090	5722	7428	15370	18547	64185	93303

Table A4.14b (continued):

TcRαβ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	744	988	606	1028	390	1374	1372	7003	266	2100
E2	115	521	42	128	0	94	368	5726	89	120
E3	207	2236	351	274	0	498	478	4302	3131	10561
E4	115	310	77	177	289	1321	431	2740	287	1020
E5	65	197	58	415	89	401	3596	21558	834	656
E6	47	362	40	368	95	329	1734	9565	923	4309
E7	36	47	84	0	32	336	710	9233	20	4255
E8	0	682	22	0	0	456	568	3218	0	508
E9	163	65	74	79	43	1587	55	1408	137	887
E10	52	156	17	0	153	845	94	994	32	587
E11	56	891	171	502	846	2276	73	3121	497	3701
E12	232	459	239	410	500	1000	191	3957	529	2080
E14	0	17	0	93	75	433	72	747	19	462
E15	0	420	21	87	59	1016	306	2821	0	826
E16	1740	1135	1920	1240	2330	3151	15049	36136	4756	6212
E17	1480	1809	0	0	617	1003	6888	14618	1163	1976
E18	522	344	1137	511	1191	1090	4635	5459	2332	2481
E20	2485	790	3686	800	2458	7032	6580	7654	28853	74039

TcRγδ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	3050	950	3451	1089	3811	1751	2312	2138	2989	1690
E2	423	124	319	156	161	280	232	824	319	466
E3	979	1365	1354	194	995	269	159	332	1102	653
E4	1362	229	1667	242	1716	502	564	223	1448	401
E5	1553	35	1840	357	1831	422	1004	1158	2125	463
E6	15155	1008	14895	1900	15505	1473	12323	2313	14108	2485
E7	4046	261	5508	675	4512	448	3497	2668	3389	5996
E8	3168	4859	2759	523	5296	2128	2210	2096	5921	7579
E9	3572	1054	4436	659	3164	1363	399	183	3048	822
E10	157	55	34	58	90	376	94	341	400	313
E11	2651	613	2508	581	1184	652	1156	1846	3724	3279
E12	1054	328	606	318	1294	339	714	1207	2403	600
E14	282	0	317	133	652	422	193	362	335	176
E15	1415	341	1199	694	606	260	667	595	2008	690
E16	5791	1213	5341	1012	2970	2181	11092	26862	9971	5306
E17	12591	1088	12038	1208	ND	ND	8610	8063	14700	2924
E18	8731	512	7487	676	8375	1433	7477	4103	5852	2311
E20	5330	828	2599	473	947	2048	6580	12079	1915	5606

TcRγδ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	911	623	944	598	1019	958	863	1412	938	1060
E2	57	63	42	104	40	245	68	696	0	430
E3	490	1171	485	166	343	202	86	143	464	653
E4	229	201	231	194	385	502	133	191	191	321
E5	65	35	116	268	59	211	141	542	98	278
E6	496	323	1830	1210	1716	941	1321	512	1134	1072
E7	708	209	642	462	608	336	760	1923	440	4391
E8	720	3096	275	286	946	1467	421	983	882	5005
E9	471	649	294	329	342	876	55	0	640	384
E10	78	55	0	39	0	251	55	209	200	268
E11	350	320	381	306	571	343	151	955	972	2056
E12	359	328	215	293	1250	304	326	1293	977	600
E14	0	0	0	133	188	375	72	280	84	176
E15	580	317	562	728	309	71	343	564	1408	402
E16	2669	1324	1500	1012	679	2018	3730	12088	5529	5824
E17	607	415	663	493	ND	ND	2419	4222	1298	2010
E18	1540	450	938	462	1957	1328	4806	5081	2341	2451
E20	2321	788	1140	405	735	2246	3792	7011	0	2637

Table A4.14b (continued):

CD4/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	37746	5456	70171	6664	56338	12320	35469	34726	50138	19564
E2	14100	3814	13247	2353	11891	1743	7449	20058	14376	3104
E3	53829	18543	49307	2157	44720	3977	17921	17896	69863	41960
E4	35326	2287	33755	1999	36973	3267	19140	12470	40860	4697
E5	27249	1117	47830	4701	28142	3317	55990	56234	48260	4050
E6	72370	4498	55087	5021	54678	5306	46258	30624	61640	23463
E7	49669	2420	73593	5602	70610	8517	44719	49671	40734	87069
E8	62160	30565	44493	4183	70289	8519	21748	24614	80894	23583
E9	24135	4860	33967	5378	18242	8111	4696	4849	20085	9871
E10	17443	1484	10509	1149	19474	9043	4497	4522	16215	3947
E11	27659	6613	24440	6981	10442	4564	11137	18100	27920	24144
E12	25153	4919	18368	3754	25814	6412	18541	19928	56156	35369
E14	15385	562	20837	2231	20948	6623	17817	12779	11876	4404
E15	110420	5333	54624	2150	56013	11288	17720	14178	110365	37619
E16	74567	4362	115969	7519	67130	7168	76739	122032	76847	10120
E17	112715	7966	84516	7861	94081	6952	81208	54023	107666	9433
E18	25354	1691	23698	1450	20719	1619	8356	4892	12425	2797
E20	41918	2717	70015	4099	39955	10159	40595	22297	193288	133470

CD4/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	20124	4029	39523	5554	33433	11480	21966	21919	32622	18800
E2	5458	2262	5275	1252	4936	1411	4621	13943	5279	2297
E3	18376	12963	16603	1348	18191	3447	10890	13205	32468	38799
E4	12132	1715	10747	1540	13740	2509	7243	6590	14676	4143
E5	9025	493	18733	2098	10489	2113	21817	28673	20206	2669
E6	23572	3345	17739	3423	21398	4211	16039	12429	29004	21094
E7	22787	1415	33435	3678	34065	6166	25458	33661	22538	73835
E8	21757	18723	15688	2218	26728	5669	9638	14525	31996	19954
E9	11188	2956	17131	3526	9520	6709	1212	2228	11353	8405
E10	5603	754	3530	718	8564	7418	2033	2386	6509	2383
E11	17529	5121	16233	5365	8043	4011	7426	15522	19986	22707
E12	20282	4271	14571	3541	23388	6045	15135	19128	45630	35061
E14	4012	375	4672	1296	8931	6008	10172	10039	5317	4002
E15	60025	4697	28368	1981	28927	10980	10958	10659	62512	36482
E16	17826	1877	37866	5336	23412	6150	28931	59097	27016	8596
E17	40782	3684	31001	4082	30003	3521	35331	33624	40475	6071
E18	16224	1230	15358	1215	13534	1584	6963	4975	7914	2638
E20	25569	1891	42831	3168	23072	8530	28609	14214	127025	110738

CD8/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	20988	3858	29874	3774	24829	4434	2035	1461	21791	3940
E2	4805	1760	3279	830	2889	898	15043	41783	3410	1042
E3	10845	6529	7307	1140	8582	1192	8195	11596	9508	3911
E4	13430	1806	10645	1341	13740	2945	9375	8688	15312	2353
E5	12707	909	17745	3877	11953	2184	28126	30135	19961	2474
E6	65608	5102	52124	5240	49986	5278	91298	70331	47725	6658
E7	21407	1470	31694	3704	27633	3237	39993	48111	17244	22986
E8	26566	21415	19789	3506	32040	4728	52135	58230	36489	9076
E9	12584	5288	18944	2824	11287	3266	8552	15528	12740	2822
E10	6934	1256	4461	1069	8049	2199	5459	6966	6152	1985
E11	10524	4698	9341	4578	23664	13686	4547	9929	13578	8909
E12	4631	1969	2976	1483	17508	5781	3136	6618	8347	3160
E14	4927	298	7345	1672	6347	2090	19273	18619	3869	1003
E15	41022	5109	19241	1844	17480	4229	36949	35236	35988	9374
E16	28788	2279	41407	3157	25994	3869	88772	173455	33196	7750
E17	43305	4603	39020	3379	37365	3875	68631	50327	43429	4314
E18	11659	1285	9391	939	10209	1454	9941	4683	10327	2579
E20	22136	1944	35856	2392	24725	7353	44653	37177	63084	22162

Table A4.14b (continued):

CD8/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	2176	1777	2380	1301	2591	2637	1156	871	2016	2480
E2	194	417	381	369	187	269	2451	10166	222	400
E3	602	2079	301	356	392	319	710	3410	522	2368
E4	1515	556	846	564	1219	1152	1112	2145	1385	1253
E5	760	257	1085	775	792	913	2270	7777	1095	728
E6	8866	2395	4673	2009	5912	2638	9000	7292	4478	4017
E7	1333	349	1318	978	1712	1364	3675	10583	1034	10448
E8	1541	3772	1231	753	1228	1565	3462	6523	1869	4558
E9	381	1639	1323	807	627	1051	358	2044	808	1390
E10	426	287	200	290	695	796	545	1094	368	678
E11	1105	1054	492	1377	4164	3769	233	2917	1041	4477
E12	870	903	687	621	3866	1843	809	4196	2067	2026
E14	97	40	96	608	113	818	734	2327	129	580
E15	6288	3034	2844	999	3206	2444	3687	6607	7064	6839
E16	5839	1621	8191	2582	5513	3265	21277	51774	10043	6934
E17	6672	2249	6711	1707	6848	2597	26953	27440	5884	2623
E18	4139	989	4362	777	4530	1378	6085	4683	6915	2715
E20	2926	807	4668	776	2515	5982	21339	17529	3953	12304

TcR Vγ9/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	2176	798	3747	743	6742	2157	59501	54527	2676	796
E2	610	193	236	0	234	102	242	409	311	32
E3	1186	1416	1070	73	1012	109	355	192	947	367
E4	815	264	1064	131	1363	315	622	386	1051	72
E5	804	18	1220	587	683	168	643	303	1128	263
E6	4468	197	2923	392	4482	253	2725	797	3498	641
E7	2137	209	3041	327	3232	270	1647	1007	2099	999
E8	2577	3336	1737	230	3071	434	1105	791	3527	1265
E9	1233	808	2010	734	1254	738	151	127	1082	498
E10	52	115	143	111	153	69	55	78	315	152
E11	1726	356	1665	569	676	104	763	436	2093	711
E12	751	338	676	269	1308	317	933	1151	2596	583
E14	282	88	220	156	314	242	566	261	270	219
E15	1323	314	828	61	689	106	500	273	1362	368
E16	1525	608	3241	1526	1475	929	4184	12952	3549	3868
E17	8297	932	5970	698	7012	1077	5244	4594	8144	1194
E18	3327	530	4149	433	3462	855	5142	3013	4367	2553
E20	2272	607	3050	517	848	3776	2005	5894	1100	11710

TcR Vγ9/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	465	345	718	357	2025	1356	8494	10331	544	465
E2	43	97	0	0	20	0	174	308	0	32
E3	94	884	134	48	212	55	159	0	58	156
E4	306	264	0	78	192	157	216	141	96	72
E5	33	18	0	84	30	101	60	230	0	52
E6	1135	157	457	143	1106	127	970	522	519	454
E7	408	80	321	197	352	210	596	745	174	606
E8	144	1858	44	115	349	81	274	348	147	807
E9	109	294	515	407	670	474	96	65	91	166
E10	26	29	34	22	27	34	35	27	0	114
E11	90	24	291	312	254	62	64	189	266	310
E12	311	184	105	197	735	269	214	975	465	524
E14	58	22	96	78	38	242	409	218	64	195
E15	742	257	265	40	368	106	269	140	254	368
E16	357	387	1650	1526	544	974	1492	6907	1835	3793
E17	1213	571	566	184	1090	502	2690	2798	962	813
E18	1031	397	1279	365	999	855	4221	3484	2647	2601
E20	1046	644	2095	962	622	3712	1473	3628	489	7886

Table A4.14b (continued):

TcR Vδ1/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	1265	471	1859	604	1887	585	2281	1915	1865	1226
E2	165	88	263	119	134	69	262	1105	171	26
E3	56	191	0	27	0	26	37	53	58	54
E4	1337	130	1257	103	1523	50	730	369	1448	397
E5	1846	18	2693	103	1534	112	2089	496	3646	212
E6	14020	705	12370	1034	13026	949	11374	2047	13224	2462
E7	1945	28	3142	209	2848	237	2813	1641	2222	3362
E8	3024	1611	2353	179	4681	1178	3462	1853	7663	9121
E9	2448	221	4338	378	3492	1085	510	774	3154	393
E10	104	58	69	0	90	77	55	75	137	41
E11	2290	839	1756	646	2093	896	763	2932	3088	3229
E12	264	96	198	42	368	97	202	486	609	465
E14	58	0	179	102	151	164	361	195	64	31
E15	650	28	478	57	427	228	797	588	946	238
E16	2026	533	2940	787	1670	1599	9146	27726	5915	5451
E17	4876	780	4175	172	3455	484	3580	4377	3938	1075
E18	4661	305	4688	423	4475	892	6792	5862	5474	2996
E20	2877	591	2864	641	1582	4456	2663	6124	2282	5677

TcR Vδ1/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
E1	84	184	85	121	126	314	154	958	359	595
E2	0	88	21	0	0	35	165	737	0	0
E3	0	191	0	27	0	26	37	53	0	54
E4	38	32	154	52	0	50	0	113	0	198
E5	33	18	0	0	0	37	0	138	49	53
E6	142	352	40	478	267	453	103	443	250	878
E7	60	28	237	119	144	27	253	906	20	1348
E8	29	487	44	67	100	413	253	522	1050	5624
E9	54	73	0	0	43	544	0	111	46	87
E10	26	0	0	0	63	77	0	0	0	0
E11	68	435	90	187	296	447	14	1294	162	1553
E12	24	96	0	42	0	37	51	325	96	351
E14	0	0	83	102	38	41	36	78	0	0
E15	46	0	21	57	0	102	195	388	254	300
E16	500	416	540	657	330	1400	2238	13815	3718	5779
E17	194	275	137	43	165	132	310	1228	134	684
E18	1072	135	796	282	1177	782	4928	6774	3214	2931
E20	1619	691	1644	570	1159	4831	1191	3192	367	3302

Table A4.15: Complete data for percentages of cellular subsets for each immune individual on a) day 0, b) day 3 and c) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)

% in gate

DONOR	R1	R2
I1	28.88	0.90
I2	32.24	1.88
I3	10.08	0.32
I4	31.64	1.76
I5	40.44	1.68
I7	71.36	3.30
I8	59.76	4.34
I9	26.08	1.44
I11	21.36	0.82
I12	83.34	4.70
I13	81.88	4.82
I14	46.46	3.14
I15	46.46	3.14
I16	75.04	9.16
I17	27.94	0.54
I18	29.58	0.90
I19	37.60	1.18
I20	84.86	4.60

CD20

DONOR	R1	R2
I1	3.94	6.12
I2	3.94	15.25
I3	6.32	34.21
I4	6.95	11.83
I5	2.85	20.59
I7	6.15	17.12
I8	6.95	17.24
I9	6.98	25.58
I11	3.18	17.86
I12	3.99	26.19
I13	5.28	21.62
I14	8.69	29.25
I15	4.39	0.00
I16	2.10	8.03
I17	1.16	15.00
I18	2.31	0.00
I19	1.03	13.33
I20	2.11	5.68

CD56

DONOR	R1	R2
I1	1.61	3.40
I2	4.36	6.77
I3	2.35	5.26
I4	2.27	7.53
I5	4.61	7.84
I7	4.95	11.67
I8	6.63	21.38
I9	6.73	16.28
I11	2.29	3.57
I12	1.18	3.96
I13	3.28	7.57
I14	1.02	4.08
I15	2.61	7.40
I16	0.77	5.11
I17	0.62	5.00
I18	1.66	0.00
I19	2.78	3.33
I20	2.60	13.64

Table A4.15a (continued):

CD56/CD3

DONOR	R1	R2
I1	1.43	1.36
I2	2.44	2.82
I3	1.23	0.00
I4	1.20	3.23
I5	0.54	0.98
I7	2.97	2.33
I8	2.21	2.07
I9	2.91	4.65
I11	0.70	0.00
I12	0.47	0.79
I13	0.76	1.62
I14	0.57	1.36
I15	0.83	3.70
I16	0.52	0.73
I17	0.21	0.00
I18	1.01	0.00
I19	0.60	0.00
I20	1.30	1.14

Total CD3

DONOR	R1	R2
I1	70.55	85.71
I2	73.87	50.28
I3	75.05	63.16
I4	71.12	51.62
I5	63.93	39.22
I7	87.10	57.97
I8	83.06	54.48
I9	61.10	29.07
I11	66.41	57.14
I12	87.17	41.27
I13	82.21	49.73
I14	60.73	43.54
I15	68.09	77.77
I16	93.97	78.10
I17	49.08	60.00
I18	52.84	47.83
I19	46.54	30.00
I20	88.96	55.69

Table A4.15b:

b)
% in gate

	RBC		PFSE	
DONOR	R1	R2	R1	R2
I1	44.00	1.78	42.80	1.72
I2	55.26	3.24	54.92	5.27
I3	28.76	0.98	20.76	0.58
I4	43.04	2.54	42.96	2.74
I5	48.32	3.74	50.36	4.46
I7	66.84	3.42	60.20	5.80
I8	53.70	4.52	48.30	5.36
I9	38.18	1.24	35.82	1.54
I11	7.66	0.66	7.56	0.46
I12	50.28	1.16	50.64	1.34
I13	60.74	3.30	60.64	2.76
I14	28.68	1.24	30.68	2.18
I15	11.74	0.52	10.60	0.36
I16	42.36	1.90	41.54	1.88
I17	17.32	0.30	18.90	0.36
I18	23.66	0.22	25.38	0.42

CD 20

	RBC		PFSE	
DONOR	R1	R2	R1	R2
I1	2.69	16.92	2.50	12.98
I2	5.17	13.87	3.26	16.39
I3	2.16	8.33	4.05	19.23
I4	1.94	3.40	1.91	8.33
I5	4.04	16.81	3.43	14.83
I7	5.01	20.78	3.62	11.39
I8	4.96	6.33	5.05	5.06
I9	2.33	9.17	1.81	13.46
I11	1.88	18.18	0.00	0.00
I12	1.76	8.06	1.59	7.61
I13	2.21	16.67	1.07	13.77
I14	3.27	14.12	3.89	21.48
I15	1.38	3.03	0.68	5.56
I16	1.44	11.50	0.99	10.31
I17	0.45	7.69	0.10	0.00
I18	1.22	0.00	1.03	0.00

CD56

	RBC		PFSE	
DONOR	R1	R2	R1	R2
I1	1.24	4.62	1.31	0.76
I2	3.02	4.01	4.31	5.25
I3	1.49	1.39	3.43	3.85
I4	1.66	0.97	5.92	8.89
I5	2.24	2.94	2.86	1.90
I7	3.32	6.93	2.92	6.40
I8	3.93	5.67	4.22	4.28
I9	4.49	10.09	3.19	3.85
I11	1.88	0.00	1.68	0.00
I12	1.42	4.03	1.16	0.00
I13	1.72	4.05	1.73	2.90
I14	2.42	2.35	4.11	4.44
I15	0.92	3.03	1.53	0.00
I16	1.00	0.00	0.55	1.03
I17	0.54	7.69	0.52	0.00
I18	2.03	0.00	1.50	0.00

Table A4.15b (continued):

CD56/CD3

	RBC		PFSE	
DONOR	R1	R2	R1	R2
I1	0.84	3.85	0.96	0.00
I2	2.05	2.19	2.92	2.95
I3	0.95	0.00	2.70	3.85
I4	1.17	0.97	5.19	8.33
I5	0.72	2.52	0.52	0.76
I7	2.46	3.90	1.74	2.49
I8	2.10	4.00	1.74	3.50
I9	3.30	8.26	2.29	3.85
I11	1.25	0.00	0.28	0.00
I12	1.02	2.42	0.77	0.00
I13	0.68	1.52	0.63	0.00
I14	1.15	2.35	2.22	0.74
I15	0.46	0.00	1.02	0.00
I16	0.86	0.00	0.40	0.00
I17	0.36	7.69	0.31	0.00
I18	1.54	0.00	0.95	0.00

Total CD3

	RBC		PFSE	
DONOR	R1	R2	R1	R2
I1	76.73	80.00	76.01	77.10
I2	81.14	63.50	81.77	70.49
I3	82.65	88.89	82.35	76.93
I4	69.89	69.42	68.06	69.44
I5	72.58	78.57	64.11	73.38
I7	89.01	70.57	88.38	80.43
I8	84.49	84.67	80.85	89.88
I9	71.56	70.65	67.50	63.47
I11	75.23	54.55	66.67	71.43
I12	88.15	76.61	90.70	79.35
I13	86.30	70.21	86.63	69.57
I14	59.61	61.17	58.08	53.33
I15	68.20	69.70	66.05	77.78
I16	93.53	76.99	93.92	75.26
I17	35.30	53.84	33.23	63.64
I18	54.87	60.00	43.29	40.00

Table A4.15c:

c)
% in gate

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	25.10	0.84	24.12	0.66
I2	46.34	3.38	45.92	4.10
I3	15.02	0.48	16.52	0.82
I4	31.46	2.00	34.20	3.02
I5	41.86	5.54	40.94	2.34
I7	57.64	3.80	55.34	3.96
I8	25.86	1.20	34.16	2.64
I9	27.26	1.24	23.02	0.72
I11	5.64	0.20	4.30	0.20
I12	43.46	1.02	44.92	0.64
I13	64.90	1.38	57.78	4.96
I14	30.12	0.52	34.06	4.14
I15	10.44	0.20	10.72	0.26
I16	35.22	0.54	34.74	0.32
I17	13.92	0.14	14.90	0.28
I18	15.74	0.20	18.44	0.38
I19	25.92	0.18	27.68	1.64
I20	56.54	1.00	58.04	1.98

CD 20

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	1.41	11.11	2.10	16.67
I2	2.38	15.33	3.21	14.08
I3	1.40	21.43	1.63	16.67
I4	1.99	12.71	1.58	7.53
I5	3.42	9.93	3.82	9.47
I7	3.61	11.11	4.45	11.18
I8	2.23	3.20	4.64	3.92
I9	1.38	3.77	2.02	0.00
I11	0.76	9.09	1.62	3.45
I12	2.00	7.41	1.75	14.86
I13	2.15	22.22	2.03	12.55
I14	2.86	24.53	3.71	18.56
I15	2.14	7.69	0.71	0.00
I16	0.75	19.23	0.73	27.91
I17	1.67	0.00	0.82	0.00
I18	0.51	0.00	0.78	7.69
I19	1.82	5.88	1.64	3.70
I20	0.23	0.00	0.39	4.00

CD56

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	0.29	1.85	0.70	2.08
I2	2.21	2.00	2.55	6.34
I3	1.08	21.43	1.09	4.17
I4	1.18	2.54	1.46	1.08
I5	0.64	0.35	2.12	2.96
I7	1.93	1.11	2.32	8.55
I8	2.24	1.60	2.89	3.92
I9	2.07	3.77	2.02	3.33
I11	5.35	9.09	3.24	3.45
I12	1.26	7.41	1.13	5.40
I13	1.02	4.94	3.61	10.59
I14	20.28	5.66	12.69	7.18
I15	1.56	0.00	2.30	5.26
I16	1.23	3.85	0.62	0.00
I17	0.91	0.00	0.98	ND
I18	1.43	0.00	1.27	3.85
I19	8.94	11.76	7.04	8.64
I20	1.80	4.00	1.18	3.00

Table A4.15c (continued):

CD56/CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	0.22	1.85	0.62	2.08
I2	1.92	1.33	1.60	4.23
I3	0.86	0.00	0.98	1.39
I4	0.81	1.69	0.90	0.00
I5	0.32	0.35	0.43	1.18
I7	1.68	1.11	1.62	3.29
I8	1.74	0.80	2.20	3.92
I9	1.69	3.77	1.63	3.33
I11	3.82	9.09	2.27	0.00
I12	1.07	0.00	0.61	1.35
I13	0.80	2.47	1.12	5.49
I14	19.10	5.66	11.34	3.59
I15	0.39	0.00	1.24	0.00
I16	1.18	3.85	0.51	0.00
I17	0.61	0.00	0.65	ND
I18	0.92	0.00	0.78	3.85
I19	4.47	11.76	2.96	2.47
I20	1.50	3.00	0.95	2.00

Total CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	75.59	77.78	77.64	77.08
I2	81.72	68.00	81.96	73.24
I3	90.86	57.14	89.11	76.39
I4	69.53	64.40	74.42	67.74
I5	74.65	86.52	76.60	82.84
I7	90.57	77.22	88.86	73.03
I8	86.04	84.80	85.26	88.23
I9	74.52	81.13	74.11	80.00
I11	75.96	72.73	68.29	86.21
I12	94.20	51.85	93.00	70.27
I13	89.49	65.43	88.28	69.41
I14	79.40	62.26	73.72	68.86
I15	78.06	84.62	74.51	84.21
I16	96.00	53.85	94.77	44.19
I17	50.31	85.71	40.91	75.00
I18	52.29	60.00	42.15	53.85
I19	41.41	23.52	41.97	69.14
I20	89.72	86.00	90.24	90.00

Table A4.16: Complete data for total numbers of cells (per 10^6 cells plated on day 0) of cellular subsets for each immune individual on a) day 0, b) day 3 and c) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (ND = not done).

a)

cells in gate

DONOR	R1	R2
I1	288800	9000
I2	322400	18800
I3	100800	3200
I4	316400	17600
I5	404400	16800
I7	713600	33000
I8	597600	43400
I9	260800	14400
I11	213600	8200
I12	833400	47000
I13	818800	48200
I14	464600	31400
I15	464600	31400
I16	750400	91600
I17	279400	5400
I18	295800	9000
I19	376000	11800
I20	848600	46000

CD20

DONOR	R1	R2
I1	11379	551
I2	12703	2867
I3	6371	1095
I4	21990	2082
I5	11525	3459
I7	43886	5650
I8	41533	7482
I9	18204	3684
I11	6792	1465
I12	33253	12309
I13	43233	10421
I14	40374	9185
I15	20396	0
I16	15758	7355
I17	3241	810
I18	6833	0
I19	3873	1573
I20	17905	2613

CD56

DONOR	R1	R2
I1	4650	306
I2	14057	1273
I3	2369	168
I4	7182	1325
I5	18643	1317
I7	35323	3851
I8	39621	9279
I9	17552	2344
I11	4891	293
I12	9834	1861
I13	26857	3649
I14	4739	1281
I15	12126	2324
I16	5778	4681
I17	1732	270
I18	4910	0
I19	10453	393
I20	22064	6274

Table A4.16a (continued):

CD56/CD3

DONOR	R1	R2
I1	4130	122
I2	7867	530
I3	1240	0
I4	3797	568
I5	2184	165
I7	21194	769
I8	13207	898
I9	7589	670
I11	1495	0
I12	3917	371
I13	6223	781
I14	2648	427
I15	3856	1162
I16	3902	669
I17	587	0
I18	2988	0
I19	2256	0
I20	11032	524

Total CD3

DONOR	R1	R2
I1	203748	7714
I2	238157	9453
I3	75650	2021
I4	225024	9085
I5	258533	6589
I7	621546	19130
I8	496367	23644
I9	159349	4186
I11	141852	4685
I12	726475	19397
I13	673135	23970
I14	282152	13672
I15	316346	24420
I16	705151	71540
I17	137130	3240
I18	156301	4305
I19	174990	3540
I20	754915	25617

Table A4.16b:

b)
cells in gate

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	140800	5696	72760	2924
I2	265248	15552	197712	18972
I3	169109	5762	14117	394
I4	180768	10668	98808	6302
I5	233869	18102	201440	17840
I7	220572	11286	210700	20300
I8	408120	34352	222180	24656
I9	83996	2728	214920	9240
I11	45960	3960	18144	1104
I12	90504	2088	111408	2948
I13	77747	4224	99450	4526
I14	77436	3348	36816	2616
I15	22071	978	27560	936
I16	169440	7600	49848	2256
I17	10392	180	22680	432
I18	33124	308	27918	462

CD 20

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	3788	964	1819	380
I2	13713	2157	6445	3110
I3	3653	480	572	76
I4	3507	363	1887	525
I5	9448	3043	6909	2646
I7	11051	2345	7627	2312
I8	20243	2174	11220	1248
I9	1957	250	3890	1244
I11	864	720	0	0
I12	1593	168	1771	224
I13	1718	704	1064	623
I14	2532	473	1432	562
I15	305	30	187	52
I16	2440	874	493	233
I17	47	14	23	0
I18	404	0	288	0

CD56

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	1746	263	953	22
I2	8010	624	8521	996
I3	2520	80	484	15
I4	3001	103	5849	560
I5	5239	532	5761	339
I7	7323	782	6152	1299
I8	16039	1948	9376	1055
I9	3771	275	6856	356
I11	864	0	305	0
I12	1285	84	1292	0
I13	1337	171	1720	131
I14	1874	79	1513	116
I15	203	30	422	0
I16	1694	0	274	23
I17	56	14	118	0
I18	672	0	419	0

Table A4.16b (continued):

CD56/CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	1183	219	698	0
I2	5438	341	5773	560
I3	1607	0	381	15
I4	2115	103	5128	525
I5	1684	456	1047	136
I7	5426	440	3666	505
I8	8571	1374	3866	863
I9	2772	225	4922	356
I11	575	0	51	0
I12	923	51	858	0
I13	529	64	627	0
I14	891	79	817	19
I15	102	0	281	0
I16	1457	0	199	0
I17	37	14	70	0
I18	510	0	265	0

Total CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	108036	4557	55305	2254
I2	215222	9876	161669	13373
I3	139768	5122	11625	303
I4	126339	7406	67249	4376
I5	169742	14222	129143	13091
I7	196331	7965	186217	16327
I8	344821	29086	179633	22161
I9	60108	1927	145071	5865
I11	34576	2160	12097	789
I12	79779	1600	101047	2339
I13	67096	2966	86153	3149
I14	46160	2048	21383	1395
I15	15053	681	18203	728
I16	158477	5851	46817	1698
I17	3668	97	7537	275
I18	18175	185	12086	185

Table A4.16c:

c)
cells in gate (10^5)

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	95456	3192	41004	1122
I2	157556	11492	183680	16400
I3	16222	518	29736	1476
I4	34606	2200	37620	3322
I5	130603	17285	80242	4586
I7	219032	14440	149418	10692
I8	41376	1920	122976	9504
I9	76328	3472	78268	2448
I11	25944	920	12040	560
I12	182532	4284	368344	5248
I13	140184	2981	145606	12499
I14	69276	1196	78338	9522
I15	9605	184	8576	208
I16	84528	1296	41688	384
I17	33408	336	35760	672
I18	23610	300	16596	342
I19	88128	612	177152	10496
I20	135696	2400	266984	9108

CD 20

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	1346	355	861	187
I2	3750	1762	5896	2309
I3	227	111	485	246
I4	689	280	594	250
I5	4467	1716	3065	434
I7	7907	1604	6649	1195
I8	923	61	5706	373
I9	1053	131	1581	0
I11	197	84	195	19
I12	3651	317	6446	780
I13	3014	662	2956	1569
I14	1981	293	2906	1767
I15	206	14	61	0
I16	634	249	304	107
I17	558	0	293	0
I18	120	0	129	26
I19	1604	36	2905	388
I20	312	0	1041	364

CD56

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	277	59	287	23
I2	3482	230	4684	1040
I3	175	111	324	62
I4	408	56	549	36
I5	836	60	1701	136
I7	4227	160	3466	914
I8	927	31	3554	373
I9	1580	131	1581	82
I11	1388	84	390	19
I12	2300	317	4162	283
I13	1430	147	5256	1324
I14	14049	68	9941	684
I15	150	0	197	11
I16	1040	50	258	0
I17	304	0	350	ND
I18	338	0	211	13
I19	7879	72	12472	907
I20	2443	96	3150	273

Table A4.16c (continued):

CD56/CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	210	59	254	23
I2	3025	153	2939	694
I3	140	0	291	21
I4	280	37	339	0
I5	418	60	345	54
I7	3680	160	2421	352
I8	720	15	2705	373
I9	1290	131	1276	82
I11	991	84	273	0
I12	1953	0	2247	71
I13	1121	74	1631	686
I14	13232	68	8884	342
I15	37	0	106	0
I16	997	50	213	0
I17	204	0	232	ND
I18	217	0	129	13
I19	3939	72	5244	259
I20	2035	72	2536	182

Total CD3

	RBC		PfSE	
DONOR	R1	R2	R1	R2
I1	72155	2483	31836	865
I2	128755	7815	150544	12011
I3	14739	296	26498	1128
I4	24062	1417	27997	2250
I5	97495	14955	61466	3799
I7	198377	11151	132773	7808
I8	35600	1628	104849	8385
I9	56880	2817	58004	1958
I11	19707	669	8222	483
I12	171945	2221	342560	3688
I13	125451	1950	128541	8676
I14	55005	745	57751	6557
I15	7498	156	6390	175
I16	81147	698	39508	170
I17	16808	288	14629	504
I18	12346	180	6995	184
I19	36494	144	74351	7257
I20	121746	2064	240926	8197

Table A4.17: Complete data for percentages of cellular subsets for each naïve individual used as a control in Ghana on a) day 0 and b) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

% in gate

DONOR	R1	R2
C1	50.9	5.7
C2	55.8	6.4
C4	51.7	9.1

CD20

DONOR	R1	R2
C1	7.6	6.2
C2	3.7	7.4
C4	7.4	7.8

CD20/CD45RO

DONOR	R1	R2
C1	0.0	0.0
C2	0.0	0.0
C4	0.0	0.0

CD56

DONOR	R1	R2
C1	0.8	4.6
C2	0.3	1.9
C4	0.5	1.3

CD56/CD45RO

DONOR	R1	R2
C1	0.0	0.2
C2	0.0	0.0
C4	0.0	0.0

Total CD3

DONOR	R1	R2
C1	80.9	68.4
C2	76.6	60.7
C4	54.9	51.6

CD45RA/CD3

DONOR	R1	R2
C1	57.5	41.0
C2	46.6	22.3
C4	23.0	15.8

CD45RO/CD3

DONOR	R1	R2
C1	13.7	30.7
C2	20.7	42.1
C4	22.2	36.9

TcRαβ/CD3

DONOR	R1	R2
C1	3.5	14.9
C2	6.3	18.9
C4	0.7	8.1

TcRαβ/CD45RO

DONOR	R1	R2
C1	0.0	0.2
C2	0.0	0.6
C4	0.0	0.0

Table A4.17a (continued):

TcRγδ/CD3

DONOR	R1	R2
C1	7.9	11.1
C2	1.3	3.7
C4	1.6	3.1

TcRγδ/CD45RO

DONOR	R1	R2
C1	0.8	2.4
C2	0.6	0.8
C4	0.6	0.6

CD4/CD3

DONOR	R1	R2
C1	42.5	31.1
C2	52.2	38.7
C4	36.0	37.4

CD4/CD45RO

DONOR	R1	R2
C1	1.8	2.5
C2	8.6	13.1
C4	6.4	5.8

CD8/CD3

DONOR	R1	R2
C1	33.3	38.2
C2	12.7	21.3
C4	16.8	17.2

CD8/CD45RO

DONOR	R1	R2
C1	0.4	1.7
C2	0.6	0.4
C4	0.4	1.0

TcR Vγ9/CD3

DONOR	R1	R2
C1	8.3	11.7
C2	0.7	1.5
C4	1.4	1.0

TcR Vγ9/CD45RO

DONOR	R1	R2
C1	0.1	0.0
C2	0.0	0.4
C4	0.0	0.1

TcR Vδ1/CD3

DONOR	R1	R2
C1	0.6	1.4
C2	0.6	3.8
C4	0.4	1.1

TcR Vδ1/CD45RO

DONOR	R1	R2
C1	0.0	0.0
C2	0.1	0.8
C4	0.1	0.0

Table A4.17b:

b)
% in gate

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	30.66	3.10	44.63	1.83	46.41	2.07	24.22	27.49	47.98	2.14
C2	65.12	1.79	65.50	1.94	59.17	7.41	22.08	21.58	50.60	12.73
C4	55.18	1.95	59.35	1.91	50.87	3.55	28.99	12.78	38.72	9.92

CD20

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	3.52	8.20	3.16	7.19	2.21	12.50	6.12	21.91	2.19	7.43
C2	8.35	27.85	8.82	19.61	4.59	9.69	1.38	2.48	2.03	4.73
C4	2.34	7.11	3.04	18.39	5.93	14.68	3.12	10.55	3.06	8.99

CD20/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.27	1.64	0.19	1.80	0.12	1.88	0.26	3.24	0.00	3.06
C2	6.27	6.33	6.18	4.58	3.21	1.21	0.82	0.43	1.43	0.35
C4	0.17	3.11	0.44	11.66	1.72	8.97	0.33	2.05	1.56	3.07

CD56

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	1.44	14.21	1.29	8.39	1.46	30.63	5.38	6.02	2.92	36.25
C2	5.20	7.59	5.69	8.50	3.42	17.36	3.59	4.89	4.62	11.37
C4	0.99	5.33	0.89	11.21	3.42	9.78	1.84	1.57	5.31	2.78

CD56/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.08	1.64	0.08	1.20	0.00	1.25	0.13	0.35	0.02	4.37
C2	3.62	3.16	3.81	3.92	1.66	1.48	0.30	0.20	0.88	1.55
C4	0.10	2.22	0.24	8.07	0.76	6.52	0.20	0.47	0.78	1.28

Total CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	53.03	56.23	47.81	56.93	48.96	49.31	62.10	88.29	48.76	37.59
C2	79.34	67.14	81.44	71.37	76.04	70.03	60.57	94.35	70.45	83.04
C4	51.47	31.29	53.35	45.19	53.45	68.36	39.25	85.12	63.16	88.03

CD45RA/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	37.82	35.34	34.32	36.84	32.49	20.74	9.04	24.65	33.69	15.60
C2	49.83	32.39	50.58	42.72	45.34	13.25	9.29	19.54	37.90	6.59
C4	18.90	8.50	19.38	15.30	17.76	13.03	4.35	19.06	16.29	5.83

CD45RO/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	14.75	27.31	11.79	26.79	13.49	35.02	33.97	67.33	11.16	26.95
C2	25.15	40.38	26.96	40.85	23.30	56.93	18.48	40.37	25.59	79.27
C4	25.17	21.09	24.97	35.23	25.16	55.59	14.85	49.96	39.64	85.43

TcRαβ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.17	1.02	0.10	2.56	0.10	2.27	0.52	5.06	0.55	3.61
C2	6.19	4.12	8.21	1.39	3.57	6.38	6.28	7.22	4.09	6.36
C4	0.67	3.39	1.05	12.73	3.79	10.88	1.64	6.65	2.76	5.54

TcRαβ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.00	0.68	0.04	1.92	0.00	2.27	0.30	4.68	0.05	2.41
C2	1.31	2.88	2.14	0.46	0.53	4.71	0.72	4.89	0.43	6.19
C4	0.00	1.69	0.08	2.73	0.22	2.92	0.00	4.72	0.21	4.85

Table A4.17b (continued):

TcRγδ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	4.99	6.08	4.18	4.03	4.40	13.55	3.01	3.28	5.71	12.27
C2	7.26	10.19	8.11	5.23	4.43	6.53	4.56	0.61	3.39	1.68
C4	1.55	7.32	2.23	12.26	4.16	10.64	1.28	1.12	3.03	2.26

TcRγδ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	1.88	6.08	1.23	4.70	1.73	13.55	2.06	3.22	1.90	11.66
C2	2.21	6.80	2.28	4.58	1.12	5.16	0.57	0.32	0.91	1.44
C4	0.52	5.28	0.44	4.72	0.68	5.32	0.14	0.48	0.72	1.29

CD4/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	23.19	36.86	29.76	33.99	26.05	35.68	19.89	51.62	27.05	35.02
C2	59.07	49.19	60.91	56.52	54.90	56.36	32.35	49.23	54.36	80.10
C4	38.30	26.50	35.95	27.61	34.62	51.32	9.40	22.86	54.11	85.54

CD4/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	7.46	13.50	10.05	18.30	8.94	26.63	11.12	37.16	8.29	29.03
C2	16.37	34.05	16.25	36.23	16.99	42.39	9.17	22.47	21.31	78.03
C4	19.24	18.00	19.73	18.18	18.04	36.36	5.39	16.79	36.82	82.12

CD8/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	21.94	24.59	22.53	20.10	19.93	23.33	38.62	60.99	22.28	18.77
C2	23.01	32.35	22.46	31.31	20.15	14.47	44.32	71.01	16.49	7.18
C4	16.18	10.78	16.65	18.86	15.16	17.50	29.57	77.54	14.04	6.66

CD8/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	1.68	7.79	1.83	6.86	1.65	14.44	4.91	15.74	1.54	11.88
C2	3.57	14.12	3.72	17.17	2.74	9.04	6.56	14.88	2.18	5.57
C4	1.76	3.35	1.91	7.02	1.71	7.50	3.67	17.37	1.64	4.25

TcR Vγ9/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	5.79	3.23	4.78	2.52	4.07	5.09	2.13	3.20	5.48	7.08
C2	5.51	8.10	6.75	5.00	3.78	3.84	4.02	0.27	1.95	0.78
C4	0.97	3.95	1.41	8.00	2.88	9.97	0.97	1.65	2.20	2.75

TcR Vγ9/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	1.08	1.79	0.78	1.26	0.96	2.31	1.33	2.84	0.84	4.58
C2	1.81	5.24	2.45	4.17	1.39	2.84	0.89	0.18	0.63	0.70
C4	0.24	3.16	0.22	3.11	0.36	4.36	0.40	0.93	0.53	1.41

TcR Vδ1/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.45	0.00	0.34	1.67	0.36	1.48	0.34	0.90	0.42	5.26
C2	4.86	6.08	9.37	8.99	2.97	2.24	4.10	0.36	2.00	0.91
C4	0.37	4.31	0.64	10.90	1.89	8.43	0.67	0.58	1.57	3.30

TcR Vδ1/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0.02	0.78	0.13	1.67	0.11	0.99	0.19	0.83	0.17	3.64
C2	1.26	3.87	2.87	5.29	0.72	1.28	1.09	0.20	0.36	0.77
C4	0.04	1.18	0.08	4.27	0.18	1.81	0.11	0.25	0.28	1.61

Table A4.18: Complete data for total numbers of cells (per 10^6 cells plated on day 0) of cellular subsets for each naïve individual used as a control in Ghana on a) day 0 and b) day 7. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli.

a)

cells in gate

DONOR	R1	R2
C1	508900	57300
C2	557900	63700
C4	516600	90600

CD20

DONOR	R1	R2
C1	38422	3570
C2	20419	4707
C4	38073	7085

CD20/CD45RO

DONOR	R1	R2
C1	0	0
C2	167	0
C4	0	0

CD56

DONOR	R1	R2
C1	4071	2630
C2	1729	1204
C4	2480	1196

CD56/CD45RO

DONOR	R1	R2
C1	102	92
C2	112	0
C4	207	0

Total CD3

DONOR	R1	R2
C1	411904	39176
C2	427519	38672
C4	283458	46741

CD45RA/CD3

DONOR	R1	R2
C1	292618	23487
C2	260149	14192
C4	118766	14315

CD45RO/CD3

DONOR	R1	R2
C1	69770	17614
C2	115653	26824
C4	114840	33459

TcRαβ/CD3

DONOR	R1	R2
C1	17557	8561
C2	35315	12033
C4	3513	7357

TcRαβ/CD45RO

DONOR	R1	R2
C1	0	120
C2	112	382
C4	103	0

Table A4.18a (continued):

TcRγδ/CD3

DONOR	R1	R2
C1	40305	6355
C2	7253	2325
C4	8421	2781

TcRγδ/CD45RO

DONOR	R1	R2
C1	4122	1387
C2	3292	516
C4	2841	580

CD4/CD3

DONOR	R1	R2
C1	216283	17803
C2	291224	24633
C4	185769	33848

CD4/CD45RO

DONOR	R1	R2
C1	9364	1433
C2	48147	8338
C4	32907	5246

CD8/CD3

DONOR	R1	R2
C1	169616	21889
C2	71076	13594
C4	86737	15565

CD8/CD45RO

DONOR	R1	R2
C1	1883	968
C2	3403	261
C4	1963	897

TcR Vγ9/CD3

DONOR	R1	R2
C1	42239	6721
C2	3850	949
C4	7336	924

TcR Vγ9/CD45RO

DONOR	R1	R2
C1	305	0
C2	167	274
C4	0	100

TcR Vδ1/CD3

DONOR	R1	R2
C1	3155	796
C2	3068	2401
C4	1963	997

TcR Vδ1/CD45RO

DONOR	R1	R2
C1	0	0
C2	391	535
C4	568	0

Table A4.18b:

b)
cells in gate

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	153300	15500	187446	7686	185640	8280	261576	296892	143940	6420
C2	86284	2372	124450	3686	125736	15746	82800	80925	69575	17504
C4	88288	3120	75671	2435	139893	9763	81172	35784	122936	31496

CD20

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	5396	1271	5923	553	4103	1035	16008	65049	3152	477
C2	7205	661	10976	723	5771	1526	1143	2007	1412	828
C4	2066	222	2300	448	8296	1433	2533	3775	3762	2831

CD20/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	414	254	356	138	223	156	680	9619	0	196
C2	5410	150	7691	169	4036	191	679	348	995	61
C4	150	97	333	284	2406	876	268	734	1918	967

CD56

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	2208	2203	2418	645	2710	2536	14073	17873	4203	2327
C2	4487	180	7081	313	4300	2734	2973	3957	3214	1990
C4	874	166	673	273	4784	955	1494	562	6528	876

CD56/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	123	254	150	92	0	104	340	1039	29	281
C2	3123	75	4742	144	2087	233	248	162	612	271
C4	88	69	182	197	1063	637	162	168	959	403

Total CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	81295	8716	89618	4376	90889	4083	162439	262126	70185	2413
C2	68458	1592	101352	2631	95610	11027	50152	76353	49016	14535
C4	45442	976	40371	1100	74773	6674	31860	30459	77646	27726

CD45RA/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	57978	5478	64331	2832	60314	1717	23646	73184	48493	1002
C2	42995	768	62947	1575	57009	2086	7692	15813	26369	1153
C4	16686	265	14665	373	24845	1272	3531	6820	20026	1836

CD45RO/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	22612	4233	22100	2059	25043	2900	88857	199897	16064	1730
C2	21700	958	33552	1506	29297	8964	15301	32669	17804	13875
C4	22222	658	18895	858	35197	5427	12054	17878	48732	26907

TcRαβ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	261	158	187	197	186	188	1360	15023	792	232
C2	5341	98	10217	51	4489	1005	5200	5843	2846	1113
C4	592	106	795	310	5302	1062	1331	2380	3393	1745

TcRαβ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	0	105	75	148	0	188	785	13895	72	155
C2	1130	68	2663	17	666	742	596	3957	299	1083
C4	0	53	61	66	308	285	0	1689	258	1528

Table A4.18b (continued):

TcRγδ/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	7650	942	7835	310	8168	1122	7873	9738	8219	788
C2	6264	242	10093	193	5570	1028	3776	494	2359	294
C4	1368	228	1687	299	5820	1039	1039	401	3725	712

TcRγδ/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	2882	942	2306	361	3212	1122	5388	9560	2735	749
C2	1907	161	2837	169	1408	813	472	259	633	252
C4	459	165	333	115	951	519	114	172	885	406

CD4/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	35550	5713	55784	2612	48359	2954	52027	153256	38936	2248
C2	50968	1167	75802	2083	69029	8875	26786	39839	37821	14021
C4	33814	827	27204	672	48431	5010	7630	8180	66521	26942

CD4/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	11436	2093	18838	1407	16596	2205	29087	110325	11933	1864
C2	14125	808	20223	1335	21363	6675	7593	18184	14826	13658
C4	16987	562	14930	443	25237	3550	4375	6008	45265	25865

CD8/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	33634	3811	42232	1545	36998	1932	101021	181074	32070	1205
C2	19854	767	27951	1154	25336	2278	36697	57465	11473	1257
C4	14285	336	12599	459	21208	1708	24003	27747	17260	2098

CD8/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	2575	1207	3430	527	3063	1196	12843	46731	2217	763
C2	3080	335	4630	633	3445	1423	5432	12042	1517	975
C4	1554	105	1445	171	2392	732	2979	6216	2016	1339

TcR Vγ9/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	8876	501	8960	194	7556	421	5572	9501	7888	455
C2	4754	192	8400	184	4753	605	3329	218	1357	137
C4	856	123	1067	195	4029	973	787	590	2705	866

TcR Vγ9/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	1656	277	1462	97	1782	191	3479	8432	1209	294
C2	1562	124	3049	154	1748	447	737	146	438	123
C4	212	99	166	76	504	426	325	333	652	444

TcR Vδ1/CD3

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	690	0	637	128	668	123	889	2672	605	338
C2	4193	144	11661	331	3734	353	3395	291	1392	159
C4	327	134	484	265	2644	823	544	208	1930	1039

TcR Vδ1/CD45RO

	CM		RBC		PfSE		PHA		PPD	
DONOR	R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
C1	31	0	244	128	204	82	497	2464	245	234
C2	1087	92	3572	195	905	202	903	162	250	135
C4	35	37	61	104	252	177	89	89	344	507

Table A4.19: Complete data for percentages of IFN-γ-stained cells on day 7 for each naïve individual. Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (arithmetic mean and SEM indicated).

% in gate

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	48.70	1.95	48.20	1.35	44.57	6.03	22.21	24.05
SO	38.97	1.30	38.42	1.29	37.63	2.69	27.90	13.04
SS	43.84	2.17	39.39	2.10	38.55	5.37	21.97	15.00

CD56

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	0.02	1.30	0.03	2.07	0.15	11.43	0.00	6.12
SO	0.03	3.97	0.08	0.96	0.11	16.16	0.06	8.85
SS	0.11	9.82	0.05	12.66	0.26	28.21	0.15	11.24

CD56/IFN-y

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	0.00	1.30	0.00	1.18	0.05	9.83	0.00	7.17
SO	0.00	3.97	0.00	1.15	0.00	16.16	0.00	10.24
SS	0.05	9.97	0.05	13.29	0.09	28.30	0.04	12.90

CD3

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	86.59	86.23	87.78	89.45	87.58	83.61	95.82	58.18
SO	94.19	89.01	94.19	93.65	92.54	79.72	97.54	79.16
SS	92.20	82.37	91.17	84.92	90.87	85.64	97.97	75.75

CD3/IFN-y

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	5.84	48.68	4.95	59.30	5.69	63.22	4.20	39.47
SO	4.04	33.68	6.32	60.54	4.04	41.76	7.23	64.51
SS	11.15	47.17	13.68	48.03	9.25	64.79	5.98	62.51

TcRαβ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	74.83	70.11	77.06	80.23	80.97	60.29	58.09	24.13
SO	79.16	59.56	80.98	71.83	74.50	57.17	65.35	62.95
SS	84.55	72.85	83.13	78.43	84.28	79.13	70.55	52.80

TcRαβ/IFN-y

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	5.08	43.84	4.78	61.60	4.67	49.51	4.36	24.44
SO	1.53	26.96	5.19	53.59	2.47	36.36	7.85	63.70
SS	9.29	58.61	8.98	57.51	8.96	68.16	5.71	53.77

TcRγδ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	0.62	2.66	0.77	4.85	0.70	16.18	0.00	9.83
SO	2.80	5.75	3.56	10.24	1.86	16.27	0.37	34.29
SS	1.50	9.40	1.59	10.76	1.15	26.40	0.20	40.02

TcRγδ/IFN-y

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	0.04	2.86	0.10	5.18	0.03	17.98	0.00	5.70
SO	0.24	6.32	0.14	11.02	0.08	17.93	0.00	26.48
SS	0.25	9.77	0.54	11.08	0.38	28.64	0.04	30.87

Table A4.20: Complete data for total numbers of IFN-γ-stained cells on day 7 for each naïve individual (per 10^6 cells plated on day 0). Resting cells (R1) and lymphoblasts (R2) are indicated for each stimuli (arithmetic mean and SEM indicated).

cells in gate

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	292200	11700	385600	10800	356560	48240	266520	288600
SO	233820	7800	307360	10320	301040	21520	334800	156480
SS	263040	13020	315120	16800	308400	42960	263640	180000

CD56

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	58	152	116	223	535	5511	0	17662
SO	70	310	246	99	331	3478	201	13841
SS	289	1278	158	2127	802	12119	395	20232

CD56/IFN-γ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	0	152	0	127	178	4742	0	20693
SO	0	310	0	119	0	3478	0	16024
SS	132	1298	158	2233	278	12158	105	23220

CD3

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	253026	10089	338467	9660	312287	40333	255375	167893
SO	220239	6943	289502	9665	278577	17155	326558	123862
SS	242510	10724	287295	14267	280243	36789	258293	136344

CD3/IFN-γ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	17050	5696	19087	6404	20288	30497	11181	113910
SO	9446	2627	19425	6247	12162	8987	24206	100937
SS	29329	6141	43108	8069	28512	27834	15752	112518

TcRαβ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	218653	8203	297143	8665	288707	29084	154808	69639
SO	185092	4645	248885	7412	224260	12303	218775	98496
SS	222400	9484	261959	13176	259920	33994	185985	95040

TcRαβ/IFN-γ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	14844	5129	18432	6653	16651	23884	11620	70534
SO	3577	2103	15952	5530	7436	7825	26282	99678
SS	24436	7631	28298	9662	27633	29282	15054	96786

TcRγδ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	1797	311	2969	524	2496	7805	0	28355
SO	6547	448	10942	1056	5599	3501	1222	53649
SS	3946	1223	4995	1808	3531	11341	514	72027

TcRγδ/IFN-γ

	CM		RBC		PfSE		SEB	
Donor	R1	R2	R1	R2	R1	R2	R1	R2
MR	117	335	386	559	107	8674	0	16450
SO	561	493	430	1137	241	3859	0	41436
SS	658	1272	1702	1861	1172	12304	105	55566

Appendix 5

Running title:

Cytokine regulation and clinical immunity to malaria in humans

Down-regulation of IL-12-dependent and IL-12-independent IFNγ production is associated with acquisition of clinical immunity to *Plasmodium falciparum* malaria[1]

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Keywords: Human, Protozoan Parasites, Inflammatory Mediators, Cytokines, T lymphocytes, NK cells.

Abstract
Individuals living in malaria endemic areas eventually develop clinical immunity to *Plasmodium falciparum*. That is, they are able to limit blood parasite densities to extremely low levels and fail to show symptoms of infection. As the clinical symptoms of malaria infection are mediated in part by pro-inflammatory cytokines it is not clear whether the acquisition of clinical immunity is due simply to the development of anti-parasitic mechanisms or whether the ability to regulate inflammatory cytokine production is also involved. We hypothesise that there is a correlation between risk of developing clinical malaria and the tendency to produce IFN-γ in response to malaria infection. In order to test this hypothesis, we have compared the ability of PBMC from malaria naive, malaria-exposed (but not clinically immune) and malaria-immune adult donors to proliferate and to secrete IFN-γ in response to *P. falciparum* schizont extract (PfSE). In order to determine how PfSE-induced IFN-γ production is regulated, we have also measured production of IL-12p40 and IL-10 from PfSE-stimulated PBMC and investigated the role of neutralising antibody to IL-12 in modulating IFN-γ production. Our results show that cells from naive donors produce moderate amounts of IFN-γ in response to PfSE and that IFN-γ production is strongly IL-12 dependent. Cells from malaria-exposed donors produce much higher levels of IFN-γ but this response is only partially IL-12 dependent. In complete contrast, cells from clinically immune donors produce minimal amounts of IFN-γ. No significant differences were detected between the groups in IL-10 production, suggesting that this cytokine does not play a major role in regulating malaria-induced IFN-γ production. The data from this study thus strongly support the hypothesis that down-regulation of inflammatory cytokine production is a component of acquired clinical immunity to malaria.

Introduction

Malaria, particularly *Plasmodium falciparum*, is responsible for more deaths than any other parasitic disease and malaria-related morbidity constitutes a significant constraint on development in tropical countries. Vaccines offer a potential means of long term malaria control but, as the clinical symptoms of malaria infection are mediated in part by pro-inflammatory cytokines (1), the design of a safe and effective vaccine requires identification of the mechanisms of anti-malarial immunity and differentiation of protective from pathogenic responses (2).

Work on murine malarias suggests that innate immune mechanisms, mediated by IFN-γ derived from natural killer (NK) cells and/or γδ T cells, limits the initial phase of parasite replication (3) and that adaptive responses (mediated by αβ T cells and B cells) are required for parasite elimination (4). IFN-γ is essential for resolution of primary infections (5). TNF-α and IFN-γ act synergistically to optimize nitric oxide (NO) production (6), which may be involved in parasite killing (7). The difference between lethal and non-lethal malaria infections can be explained, in part, by the ability of mice to mount an early IL-12, IFN-γ or TNF-α response (8-11). However, IFN-γ also plays a role in the acute symptoms of infection, such as fever, anorexia and weight loss, through induction of TNF-α and other endogenous pyrogens. Over-production of IFN-γ or TNF-α predisposes to severe pathology (11-13) and, in lethal infections, neutralisation of IL-12, blocking the IFN-γ pathway or down-regulation of TNF-α by administration of rTGF-β abrogates mortality (12,14-16).

In humans, sterilising immunity to malaria rarely, if ever, develops but individuals living in endemic areas become clinically immune; i.e. they are able to control circulating parasite densities to within tolerable limits and show no significant clinical symptoms of infection. Although IFN-γ responses to some defined malaria antigens correlate with protective immunity (17,18), plasma IFN-γ concentrations are higher in symptomatic than in asymptomatic infections (19) and there is a temporal association between IFN-γ secretion and fever (20). Similarly, plasma TNF-α and nitrogen oxide levels are associated with rapid resolution of fever and parasite clearance (21,22) but severe *P. falciparum* malaria is accompanied by high levels of circulating cytokines, including TNF-α (23-26). Taken together, these observations suggest that there is a critical balance to be found in the inflammatory response to malaria infection and that the ability to regulate cytokine levels, within quite precise limits, may be a component of clinical immunity.

We have previously shown that although peripheral blood mononuclear cells (PBMC) from malaria-immune African adults make strong IFN-γ responses to certain purified malaria antigens, IFN-γ responses to crude parasite extracts are low or non-existent (27). Similar findings have been reported elsewhere (28). In contrast, cells from malaria-naive, European adults (who are at high risk of developing severe malaria if infected) produce significant amounts of IFN-γ after stimulation with *P. falciparum* schizont extracts (PfSE) or live malaria parasites (29,30). Although the majority of cells being activated in naive donors are TCRαβ+ CD45RO+ or TCRγδ+ Tcells (29,30), the source of the IFN-γ has not been determined. The basis for this strong recall antigen response appears to be priming of malaria-reactive, memory T cells by exposure to cross-reacting antigens expressed on a wide variety of commensal organisms and common pathogens (31,32).

We hypothesise that there is a correlation between the risk of developing clinical malaria and the ability to produce IFN-γ in response to malaria infection, and that this can be assessed in vitro as the ability of PBMC to produce IFNγ when reactivated by PfSE. In order to test this hypothesis, we have compared the ability of PBMC from malaria naive, malaria-exposed (but not clinically

immune) and malaria-immune adult donors, to proliferate and to secrete IFN-γ in response to PfSE stimulation in vitro. In order to determine how PfSE-induced IFN-γ production is regulated, we have also measured production of IL-12 and IL-10 from PfSE-stimulated PBMC and investigated the role of neutralising antibody to IL-12 in modulating IFN-γ production.

Subjects and Methods

Subjects

Malaria naive donors (n = 19, aged 20-41 years, 11 male, 8 female) were recruited from Edinburgh, Scotland, UK. None of the donors had knowingly been infected with malaria and the majority had never visited a malaria-endemic area. Malaria-immune donors (n = 20, aged 30-47 years, 8 male, 12 female) were recruited from the village of Dodowa, approximately 40 miles inland from Accra, Ghana, where malaria transmission is highly endemic (33) and the majority of adults show no clinical symptoms when infected with malaria. Malaria-exposed donors (n = 20, aged 21-60 years, 17 male, 3 female) were recruited from the Greater Accra district where levels of malaria transmission are much lower than in rural areas (33) and many adults still develop clinical symptoms when infected by malaria.

A questionnaire was completed for each donor, giving information on prior exposure to malaria and use of anti-malarial drugs. Up to thirty mls of venous blood were collected into heparinised containers. An aliquot of plasma was removed for serology and Giemsa-stained blood films from all the African donors were examined for the presence of malaria parasites. A highly sensitive PCR method, based on the multicopy 7H8/6 gene sequence (34), was used to detect very low level *P. falciparum* infections. Details of the PCR method are given elsewhere (35).

P. falciparum schizont antigens

P. falciparum clone 3D7 (36) was maintained in continuous culture (37). Cultures were routinely screened for mycoplasma using a commercial PCR kit (BioWhittaker, Wokingham, UK) and shown to be free of contamination. When parasitaemia reached approx 6-8%, mature schizonts were separated on a 60% Percoll gradient (Pharmacia, Uppsala, Sweden), washed three times in serum-free RPMI 1640 (Gibco, Paisley, UK), pelleted by microcentrifugation and resuspended at a concentration of 10^8 infected erythrocytes per ml. The suspension was freeze-thawed three times by immersion in liquid N_2 .The entire extract (designated PfSE) was used in cell culture experiments. A portion of the extract was spun to remove the cellular debris; the supernatant was saved and used for serology (designated soluble *Pf* antigen). Both antigen preparations were aliquoted and stored at -80°C until required. Freeze thaw preparations of uninfected erythrocytes (10^8/ml) (uRBC) were used as controls.

Serology

Plasma from all donors was tested for anti-malarial antibodies by ELISA. Immulon IV microtitre plates (Dynex, Billingshurst, UK) were coated with an optimal concentration of soluble *Pf* antigen (determined by titration) diluted in carbonate/bicarbonate buffer and incubated overnight at 4°C. Plates were blocked for 3 hours at room temperature with PBS containing 0.5% Tween 20 (Sigma, Poole, UK) and 1% non-fat milk powder (blocking buffer). Plasma samples, diluted 1:1000 in blocking buffer, were added to duplicate wells, and incubated for 3 hours at RT. Bound antibody was detected with rabbit anti-human IgG-horse radish peroxidase (Dako, High Wycombe, UK) and *o*-phenylenediamine/H_2O_2 as described previously (35). Optical density was measured at 492nm. Samples were designated positive for anti-malarial antibody if the OD was greater than the mean plus 2SD of the OD values of 22 control European plasmas. Ab levels are expressed as a % of the value obtained for a pool of hyperimmune plasma from African adults tested on the same plate.

Cell cultures

Mononuclear cells were separated from heparinised blood by centrifugation through Lymphoprep® (Pharmacia, Uppsala, Sweden), washed in RPMI 1640 and resuspended at a concentration of 10^6 viable cells per ml in complete culture medium (RPMI 1640 with 2mM L-

glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, 30mM HEPES and 0.22% (v/v) sodium bicarbonate; all Sigma) with 10% heat-inactivated, non-immune human serum. For lymphocyte proliferation assays, cells were aliquoted (100µl/well) into sterile, round-bottomed, 96 well microtitre plates. Antigens, at optimal concentration in complete medium, or the mitogen PHA (2 µg/ml, Sigma) were added to triplicate wells and plates incubated at 37°C in 5% CO_2 for 2,4,6 or 8 days. Eighteen hours before harvesting, 100µl of supernatant was removed from each well and reserved for cytokine analaysis. 1µCi ^{3}H- thymidine (Amersham, UK) in 100µl fresh culture medium was added to each well. Cells were harvested onto cellulose filters and incorporation of radio-labelled thymidine assessed by scintillation counting. The geometric mean counts per minute (cpm) were determined for each antigen and the stimulation index (SI) calculated as the ratio of PfSE-stimulated cpm to control (uRBC-stimulated) cpm. SI values ≥ 2.5 were considered positive (38).

In order to determine the interaction between IFN-γ production and IL-12, a neutralising antibody to human IL-12 (R&D Systems, Abingdon, UK)) or a control, isotype-matched murine IgG (R&D Systems) were added to the cultures at a final concentration of 5 µg/ml. Antibodies were added at the beginning of the culture period (2, 4 or 6 days) and remained throughout. Supernatants were collected and tritiated thymidine incorporation assessed as described above.

For intracellular cytokine staining, 2 x 10^6 PBMC were aliquoted into each well of a 24 well, flat-bottomed culture plate and incubated for 7 days. PfSE or uRBC were added to duplicate or triplicate wells at the concentrations given above; the superantigen staphylococcal enterotoxin B (SEB) (2 µg/ml, Sigma) was used as a positive control.

Cytokine assays

Cell culture supernatants were tested for presence of IFN-γ, IL-12p40 and IL-10 by two site capture ELISA. Flat-bottomed, 96 well microtitre plates (Immulon IV) were coated overnight with 100µl per well of an optimal concentration of capture antibody diluted in PBS. Plates were washed 4 times with PBS plus 0.05% Tween 20, blocked with 200 µl/well blocking buffer (PBS plus 4% bovine serum albumin, Sigma) for 1 hour at 37°C and washed three more times. Culture supernatants or cytokine standards (100µl/well) were added to duplicate wells and incubated at room temperature for 3 hours. After washing four times, 100µl /well of biotinylated capture antibody (diluted in PBS) was added and incubated for 1 hour at room temperature. Plates were washed 6 times, avidin-labelled horse-radish peroxidase (1 µg/ml, Sigma) added for 30 minutes at room temperature and after a final 8 washes, plates were developed for 15 minutes at room temperature with hydrogen peroxide as substrate and *o*-phenylenediamine as chromagen. The reaction was stopped with 2M H_2SO_4 and absorbance read at 492 nm.

Antibody pairs for IL-10 and IFN-γ, and all cytokine standards, were obtained from Endogen, Woburn, MA, USA. The anti-IL-12 antibody pair was obtained from R&D systems. Coating antibodies for IL-10 and IFN-γ were used at 2 µg/ml, coating antibody for IL-12p40 was used at 4 µg/ml. Biotinylated antibody to IL-10 and IFN-γ were used at 0.05µg/ml, antibody to IL-12p40 was used at 0.3 µg/ml.

Cytokine concentrations were calculated from standard curves using best fit formulae. The lower limit of detection (LLD) was defined as 2 SD above the mean of the negative control (culture medium alone). Limited volumes of culture supernatants precluded the evaluation of additional cytokines.

Intracellular cytokine staining

Eighteen hours prior to staining, 3μg/ml Brefeldin A (Sigma) was added to cell cultures to block secretion of cytokine from the endoplasmic reticulum. Cells were washed with PBS, resuspended at $2x10^6$ cells/ml in PBS with 1% foetal bovine serum (FBS, Sigma) and 0.1% NaN_3, and double-stained with labelled antibodies to CD3 (tricolour) and either CD56 (PE), TcRαβ (FITC) or TcRγδ (FITC) (all from T Cell Sciences, Claydon, UK). Cells were incubated at 4°C for 40 minutes, washed in PBS, resuspended in 300μl PBS with 4% paraformaldehyde (BDH, Poole, UK) and incubated in the dark, at room temperature for 15minutes. After washing, cells were resuspended in 300μl PBS with 1% FBS, 0.3% saponin (BDH) and 0.1% NaN_3 containing 2 μg of anti-IFN-γ antibody, labelled with either FITC (TCS) or PE (Becton-Dickinson, London UK), and incubated in the dark for 30 minutes at 4°C. Finally, cells were washed once in saponin/PBS and resuspended in 0.5ml PBS for analysis by three colour flow cytometry (FACscan, Becton-Dickinson).

Statistical methods

Differences between groups were assessed by Student's t test on log-transformed data. Differences in response to different treatments within a group, were assessed by paired t tests comparing treated and control cells from the same donors. For antigen-specific responses, where background or uRBC values were subtracted and thus some negative values were obtained, the non-parametric Mann Whitney U test was used.

Results

The three groups of donors were clearly distinguishable on the basis of their clinical history, malaria infection rates and serological responses to soluble malaria antigens (Table I). The naive donors had no previous history of malaria infection and antibody levels were within the normal range defined by non-immune sera. The majority of the exposed but non-immune donors reported a clinical malaria infection (confirmed by microscopy and treated with anti-malarial drugs) within the last five years. Only one of the donors had a subclinical malaria infection at the time of blood sampling. Several donors had anti-malarial antibody levels below the cut-off level defined by non-immune sera and the mean antibody titre was only 41% of the hyperimmune control serum. In contrast, in the clinically immune group, only one donor reported a confirmed clinical malaria attack in the past five years, 8 donors were subclinically infected at the time of sampling (two detected by blood film, and 6 by PCR), all donors were seropositive and mean antibody titers were 87% of the hyperimmune control.

Lymphoproliferative responses

Proliferative responses were assessed after 3, 5 and 7 days. Cells from all donors proliferated strongly to PHA, showing that cells were viable (data not shown). In all three groups, the geometric mean SI for PfSE/uRBC increased steadily with time (Figure 1), but there was no significant difference in the mean SI between the groups. The only noticeable difference between the groups was that responses appeared slightly earlier in the exposed group than in the other two groups (cells from 13/19 donors gave an SI ≥ 2.5 at day 2 compared to 6/19 naives and 8/18 immunes).

IFN-γ production

IFN-γ in cell supernatants was measured after 2, 4 and 6 days (Figure 2). For naive donors, IFN-γ levels in cultures stimulated with PfSE rose steadily over time, reaching a geometric mean value of 224 pg/ml at day 6. IFN-γ levels in PfSE cultures were significantly higher than in uRBC cultures on days 4 and 6 (paired $t \geq$, $df = 18$, $P < 0.002$). For malaria-exposed donors, PfSE-specific IFN-γ levels rose to a mean of > 900 pg/ml at 6 days, were significantly higher than in uRBC-stimulated cultures on days 2, 4 and 6 (paired $t \geq 3.46$, $df \geq 17$ $P < 0.003$ on all days) and were significantly higher than for naive donors at both 4 and 6 days (Mann-Whitney $U = 253$, $df = 35$, $p < 0.001$). In contrast, for immune donors, geometric mean IFN-γ levels increased only marginally over time and the difference between uRBC-stimulated and PfSE-stimulated cultures was minimal. Malaria specific IFN-γ levels were significantly lower in immune donors than in either the naive or the exposed group at both day 4 and day 6 (Mann-Whitney U, $W > 230$, $df > 29$, $P < 0.008$ at day 4, $P < 0.001$ at day 6).

However, despite the clear differences between the groups in mean IFN-γ levels, it is worth noting that not all naïve donors cells produced IFN-γ and that a proportion of "clinically-immune" donors cells did produce IFN-γ, albeit transiently and at low levels. The number of IFN-γ responders (defined as donors whose cells produced at least twice as much IFN-γ in PfSE-stimulated cultures as in uRBC cultures on at least one of the days tested) was highest for exposed donors (19/20, 95%), intermediate for naïve donors (14/19, 74%) and lowest for immune donors (5/20, 25 %).

IL-12 production

IL-12 p40 was measured by ELISA in 1, 2, 4, 6 and 8 day supernatants of PfSE-stimulated cultures. As IL-12 levels peaked at day 2 in all donors, only day 2 data is shown (Figure 3). Overall, levels of PfSE-specific IL-12 in supernatants were very low. Cells from exposed donors produced up to 530 pg/ml PfSE-specific IL-12, but levels varied widely between individuals.

Median IL-12 levels were not significantly different between the groups and values obtained for PfSE-stimulated cells were not significantly different from values for uRBC-stimulated cultures (paired t tests in all 3 groups). However, when individuals are classified as either responders or non-responders (where a responder is defined as PfSE IL-12 – uRBC IL-12 > 20 pg/ml) then only 11% of naïve donors (2/18) were responders but 45% (9/20) of exposed and 35% (7/20) of immune donors responded.

Relationship betwen IL-12 and IFN-γ production.

When IL-12 and IFN-γ production were compared in individual donors it was clear that the peak in IL-12 production preceded the rise in IFN-γ production; IL-12 levels tended to peak at day 1 or day 2 whilst IFN-γ levels typically began to increase at day 4 (data not shown). To determine whether there was a causal association between IL-12 and IFN-γ levels, neutralising antibodies to IL-12 (or control, isotype-matched antibodies) were added to PfSE-stimulated or uRBC-stimulated cell cultures and IFN-γ levels were measured (Figure 4).

Addition of anti-IL-12 or control IgG had no significant effect on lymphoproliferative responses (data not shown), but reduced IFN-γ production in all groups of donors.

As described above, cells from naive donors produced modest, but significant, amounts of IFN-γ. In the presence of neutralising antibody to IL-12, IFN-γ levels were reduced to background levels (Fig 4a), indicating that IFN-γ production from naive cells is IL-12 dependent. Differences in IFN-γ production between anti-IL-12 treated and control cultures were significant on days 2, 4 and 6 but the effect was most marked at day 6 (paired $t = 3.05$, $df = 13$, $P = 0.009$). Cells from exposed donors produced much higher levels of IFN-γ than cells from naïve donors and in the presence of anti-IL-12, IFN-γ levels were also significantly reduced at all time points (paired $t \geq 3.41$, $df = 19$, $P \leq 0.003$ on all days) (Fig 4b). However, anti-IL-12 did not completely inhibit IFN-γ production and at day 6 IFN-γ levels were still significantly higher than in unstimulated cultures ($t = 5.2$, $df = 16$, $P < 0.001$) or in PfSE-stimulated cultures of naive cells ($t = 4.56$, $df = 32$, $P < 0.001$). These data indicate that, in malaria-exposed donors, IFN-γ production may derive from more than one cellular source, only one of which is IL-12 dependent. Cells from malaria-immune donors made very little IFN-γ, nevertheless the addition of anti-IL-12 reduced IFN-γ levels to background values and the difference between control and anti-IL-12 cultures was marginally significant at day 6 (paired $t = 2.18$, $df = 19$, $P = 0.042$) (Fig 4c).

Cellular source of IFN-γ

In order to determine the cellular source of IFN-γ in PfSE-stimulated cell cultures, cells from three naïve donors were cultured for 7 days in the presence of PfSE, uRBC, or (as a positive control) the superantigen SEB, stained for cell surface markers CD3, CD56, TcRαβ and TcRγδ, permeabilised and counterstained for intracellular IFN-γ. Labelled cells were analysed by three colour flow cytometry. Resting lymphocytes and lymphoblasts were gated and analysed separately. As can be seen from Figure 5, the majority of the IFN-γ positive cells were in the lymphoblast population, so these cells were examined in detail (Table II). In all donors, the proportion and absolute number of lymphoblasts was higher in PfSE-stimulated cultures than in control uRBC cultures (mean, SD, $4.70 \pm 1.77\%$ compared with $1.58 \pm 0.45\%$). Of the PfSE-induced lymphoblasts, 11-28% were CD3-, CD56+ (i.e. NK cells) and 85-100% of these cells were also positive for IFN-γ. Sixty to 80% of lymphoblasts were TCRαβ+ and 70-95% of these cells were IFN-γ+. Sixteen to 26% of lymphoblasts were TCRγδ+ and 100% of these cells were IFN-γ+. Thus, the IFN-γ-producing cells appear to be a mixed population of NK cells, αβ T cells and γδ T cells with approx 60% of the cells being αβ T cells.

IL-10 production

The finding that cells from clinically immune individuals produce minimal amounts of IFN-γ in response to malaria antigen stimulation is consistent with our hypothesis that clinical immunity to malaria is associated with down-regulation of the inflammatory cytokine response, but does not tell us how this down-regulation occurs. Also, the fact that cells from malaria-exposed individuals produce markedly higher levels of IFN-γ than naïve donors, but are not obviously at greater risk of severe malaria, suggests that they may have developed mechanisms for ameliorating the impact of these high IFN-γ levels. Therefore, we measured levels of the anti-inflammatory cytokine IL-10 in cell supernatants to determine whether differences in IL-10 production might explain either the down-regulation of the IFN-γ response or the ability of exposed individuals to avoid severe consequences of malaria in the presence of a strong pro-inflammatory response.

Overall, levels of malaria-specific IL-10 were extremely low in all cultures on all days. There were no significant differences between the three groups in mean IL-10 levels (Table III) but when individuals were classified as either IL-10 responders or non-responders to PfSE, the highest number of responders was seen in the exposed group and the lowest number of IL-10 responders was seen in the immune group. This same pattern was observed in PHA-induced IL-10 responses, with exposed donors producing the highest levels of IL-10 and immune donors the lowest.

Discussion

Clinical immunity to malaria is characterised by the ability to limit blood parasite densities to extremely low levels and by the failure to show symptoms of infection. The mechanisms by which parasite numbers are controlled are not entirely clear but recent evidence supports a role for antibody-dependent cell-mediated inhibition of parasite growth (ADCI), which involves both parasite-specific antibodies and the release of inflammatory cytokines from monocyte/macrophages (39). The clinical symptoms of malaria are related to the presence of circulating pro-inflammatory cytokines such as TNF-α (1) but the risk of clinical disease also increases with increasing parasite density (40). It is therefore not clear whether the acquisition of clinical immunity is due simply to the development of anti-parasitic mechanisms which keep parasite densities below a critical threshold or whether the ability to regulate inflammatory cytokine production is also involved. One piece of clinical evidence that points to the importance of the latter is that parasite densities which are well tolerated by immune individuals can be accompanied by severe febrile symptoms in non-immunes (40).

For many years it was believed that glycophospholipid components of the parasite, possibly derived from the membrane anchors of surface antigens (41), directly induced macrophages to secrete TNF-α (42,43) but the notion that this is sufficient to mediate the pathology of malaria has recently been questioned (44). We have postulated that IFN-γ is required to induce sufficiently high levels of inflammatory mediators to cause disease and that the ability to down-regulate IFN-γ production (or its effects) may be a pre-requisite for clinical immunity (32). To date, this hypothesis is supported only by two small studies showing that levels of malaria-induced IFN-γ are lower in African adults than in European adults living in Africa (27,28).

In this study, we have directly addressed the relationship between clinical immunity to malaria and the tendency of PBMC to produce either pro-inflammatory or anti-inflammatory cytokines in response to stimulation with a crude preparation of malaria antigens – an antigen preparation which we believe closely represents the pool of circulating antigen released into the peripheral circulation at the time of schizont rupture. The selection of peripheral blood as the source of leucocytes is dictated by practical constraints but has been validated in many previous studies: although malaria-reactive T cells tend to disappear from the peripheral circulation during an acute infection (probably migrating to the spleen and liver) they are released back into the periphery upon resolution of the infection (45) and it is thus realistic to assume that, in healthy individuals, the peripheral T cell population is representative of the total malaria-reactive T cell pool.

Subjects were allocated to naive, exposed or immune groups according to their place of residence and before any laboratory analyses (parasite detection, serology or cellular assays) were performed. Parasite detection and serology confirmed our allocation of individuals to appropriate groups, with the possible exception of one person in the exposed group (who had a low density, asymptomatic infection at the time of sampling and might therefore be regarded as clinically immune) and one person in the immune group (who reported a clinical attack of malaria 9 months previously and might therefore be considered exposed but non-immune). However, to avoid selection bias, neither of these subjects were reallocated to alternative groups.

The most marked differences in cellular immune responses between the groups were in the amounts of IFNγ produced following in vitro stimulation with PfSE. The low mean levels of IFN-γ production by cells from immune donors (and the complete lack of malaria-induced IFN-γ production by 75% of these donors) strongly supports the hypothesis that clinical immunity to malaria is accompanied by down-regulation of the pro-inflammatory immune response.

The mechanism by which IFN-γ levels are down-regulated in immune donors is not clear. Direct antagonism by IL-10 seems unlikely given the failure to detect significant levels of IL-10 in PfSE-stimulated cell supernatants; another recent study also failed to find significant induction of IL-10 from PBMC by *P. falciparum* extracts (46). Plasma levels of IL-10 are raised during clinical malaria episodes, and are higher in severe cases than in uncomplicated cases (47-49) but levels are not raised in children with asymptomatic malaria infections, suggesting that IL-10 is upregulated in parallel with pro-inflammatory cytokines but that it does not, in fact, play a major anti-inflammatory role.

The lack of detectable cytokine production in response to PfSE in immune individuals is not due to T cell anergy; PBMC from immune individuals proliferated in response to PfSE to the same extent as cells from other donors and thus, presumably, produce IL-2. It remains to be seen whether these proliferating cells produce any other cytokines which may down-regulate inflammatory responses. A prime candidate for such a cytokine would be TGF-β: we have recently shown a causal association between low TGF-β levels and severity of malaria in mice (16) and the plasma of acute *P. falciparum* patients contains lower than normal levels of circulating TGF-β (50). A potential mechanism of TGF-β action is down-regulation of IL-12-mediated IFN-γ induction by inhibiting expression of the IL-12 receptor on T cells (51). Although we could not detect significant levels of free IL-12 in most PfSE-stimulated PBMC supernatants, and IL-12p40 mRNA could not consistently be detected by RT-PCR (data not shown), neutralisation of IL-12 with antibody had a significant effect on IFNγ production, demonstrating that this cytokine does play a role in the inflammatory cytokine response to malaria parasites.

Cells from naïve donors produced moderate levels of IFN-γ, commensurate with the known risk of non-immune adults developing clinical malaria during their first infection. The IFN-γ response appeared to be strongly IL-12 dependent suggesting that NK cells may be the prime source of IFN-γ in these donors. However, there was some variation between donors in the effects of anti-IL-12 and in some naïve individuals anti-IL-12 only partially blocked IFN-γ production. Intracellular cytokine staining of PfSE-activated PBMC's from naïve donors indicated that IFN-γ is produced by a mixed population of blasting cells, predominantly αβ T cells but with significant numbers of NK cells and γδ T cells. These data correlate well with previous observations that in naïve donors PfSE activates mainly CD3+ TCRαβ+ cells, that γδ+ T cells can also be activated under certain circumstances and that both populations can produce IFN-γ (29-31,52). The αβ T cells are believed to have been primed by cross-reacting antigens present in a number of commensal organisms and common pathogens (53) whereas γδ T cells recognise phosphorylated non-protein antigens (54). Cytokine production by malaria-activated human NK cells has not previously been reported, although activation of NK cells by during malaria infection has been observed (55).

Interestingly, cells from malaria-exposed donors produced very much higher levels of IFN-γ than naïve donors and this response was only partly IL-12-dependent. The most plausible explanation for this would be that malaria-exposed donors possess an additional population of IFN-γ-producing T cells, specifically primed by malaria antigens rather than cross-reacting antigens. Further experiments are required to determine the cellular source of IFN-γ in this population. Despite producing very high levels of IFN-γ in response to malaria antigens, exposed donors are not expected to be at any greater risk of severe malaria than naïve donors and their risk may indeed be lower. This suggests that the downstream effects of IFN-γ production – for example induction of TNF-α – may be being inhibited to avert serious pathology. As in immune donors,

malaria antigens did not appear to induce significant levels of IL-10 (although cells from almost half the exposed donors did produce some IL-10 after stimulation with PfSE) but other cytokines, such as TGF-β, may a play role in controlling the down-stream effects of IFN-γ. In mice infected with malaria, TGF-β inhibited TNFα production without any significant effect on IFN-γ levels (16).

In summary, we have shown that there is strong association between IFN-γ production by PBMC in response to malaria antigen stimulation and the immune status of the donor. Specifically, PBMC from malaria-immune individuals, who are able to resolve malaria infections without developing clinical disease, produce negligible levels of IFN-γ. In contrast both T cells and NK cells from non-immune individuals produce moderate levels of IFN-γ, in an IL-12-dependent manner. Intriguingly, the highest levels of IFN-γ were observed in partially-immune donors who develop clinical symptoms of infection but are at relatively low risk of severe disease. Further studies are required to elucidate the mechanisms by which IFN-γ production is down-regulated in malaria-immune individuals.

Acknowledgements:

We would like to thank the people of Dodowa and the staff of the Noguchi Memorial Institute for Medical Research for their participation in these studies. We thank Kevin Tetteh for parasite diagnosis by PCR, Enid Owusu, Michael Addae, Michael Ofori , Ben Gyan and Andrew Sanderson for technical assistance.

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Figure legends

FIGURE 1: Lymphoproliferative responses

Lymphoproliferative responses to *P. falciparum* schizont extract (PfSE) in PBMC from malaria naïve (■), exposed (▲) and immune (O) donors. Geometric mean SI (PfSE/uRBC), 95% CI.

FIGURE 2: Interferon-γ production

PfSE-specific (PfSE-uRBC) IFN-γ production (pg/ml) by PBMC from malaria naïve (■), exposed (▲) and immune (●) donors (median and 75th percentile).

FIGURE 3: IL-12 p40 production

PfSE-specific (PfSE-uRBC) IL-12 p40 (pg/ml) in 48 hour PBMC supernatants from malaria naïve, exposed and immune donors. Figures indicate median values for each group.

FIGURE 4: Effect of neutralising antibodies to IL-12 on IFN-γ production.

Effect of neutralising antibodies to IL-12 (5μg/ml) on PfSE-induced IFN-γ production (pg/ml) from PBMC from (a) naïve, (b) exposed and (c) immune donors. (Mean, upper 95% CI).

FIGURE 5: Intracellular staining for IFN-γ

Intracellular cytokine staining to determine the cellular source of IFN-γ in PBMC from a malaria naïve donor cultured for 7 days with either SEB (panels a,d,g), PfSE (b,e,h) or uRBC (c,f,i). Lymphocytes were divided into resting cells (R1) and lymphoblasts (R2) on the basis of forward and side scatter (panels a to c) and. and stained for CD3 (vertical axis) and IFN-γ (horizontal

axis) (panels d-i). Few IFN-γ+ cells were seen in the resting population (panels d-f) but IFN-γ+ cells were seen in the blasting population (panels g-i). In PfSE-stimulated cultures, IFN-γ+ cells were predominantly CD3+ but some CD3- cells were also IFN-γ+.

Table I: *Details of blood donors*

	Naive (UK)	Exposed (Accra)	Immune (Dodowa)
N	19	20	20
Age range (years)	20-41	21-60	30-47
Pf Blood film + (%)	0 (0%)	0 (0%)	2 (10%)
Pf PCR + (%)	0 (0%)	1 (5%)	6 (30%)
Antibody + (%)	0 (0%)	13 (68%)	20 (100%)
Mean Ab* (SEM) Range	0.05 (0.004) 0.04 – 0.08	0.41 (0.06) 0.13 – 1.01	0.87 (0.08) 0.24 – 1.43
Confirmed clinical malaria in the last 5 years? (%)	0 (0%)	14 (70%)	1 (5%)

*Individual Ab OD values are expressed as a ratio of the value of a high titre pool of malaria immune serum from African donors.

Table II: *Phenotyping of IFN-γ-secreting cells by flow cytometry.*
Absolute numbers of lymphoblasts (x 10^{-3}) after 7 days stimulation of 2 x 10^6 PBMC from malaria naïve donors. (Mean ± SE, n = 3).

	PfSE	uRBC	SEB
Total	38 ± 8.1	13 ± 2.3	208 ± 40.5
CD3+	31 ± 6.9	11 ± 1.7	143 ± 23
CD3+ IFN-γ+	22 ± 6.9	7 ± 0.6	109 ± 4.1
CD56+	7 ± 2.3	1 ± 0.6	17 ± 1.7
CD56+ IFN-γ+	7 ± 2.3	1 ± 0.6	20 ± 2.3
TCR αβ+	25 ± 6.4	10 ±1.7	88 ± 9.3
TCR αβ+ IFN-γ+	20 ±6.4	7 ±1.2	89 ± 9.3
TCR γδ+	8 ± 2.3	1 ± 0.6	51 ± 12.7
TCR γδ+ IFN-γ+	8 ± 2.3	1 ± 0.6	38 ± 11.6

Table III: *IL-10 concentration (pg/ml) in PBMC supernatants after 2 days in culture.*

		uRBC	*PfSE*	*PHA*
Naïve (n = 19)	***Mean***	68.7	68.4	194.7
	SE	10.5	9.3	28.0
	responders %		21.1	100.0
Exposed (n = 20)	**Mean**	74.1	70.8	201.5
	SE	8.9	7.7	33.4
	responders %		45.0	95.0
Immune (n = 18)	**Mean**	41.5	43.2	70.3
	SE	0.5	1.5	7.3
	responders %		11.1	94.4

Responder: PfSE-induced IL-10 ≥ 5 pg/ml higher than control (uRBC) value.

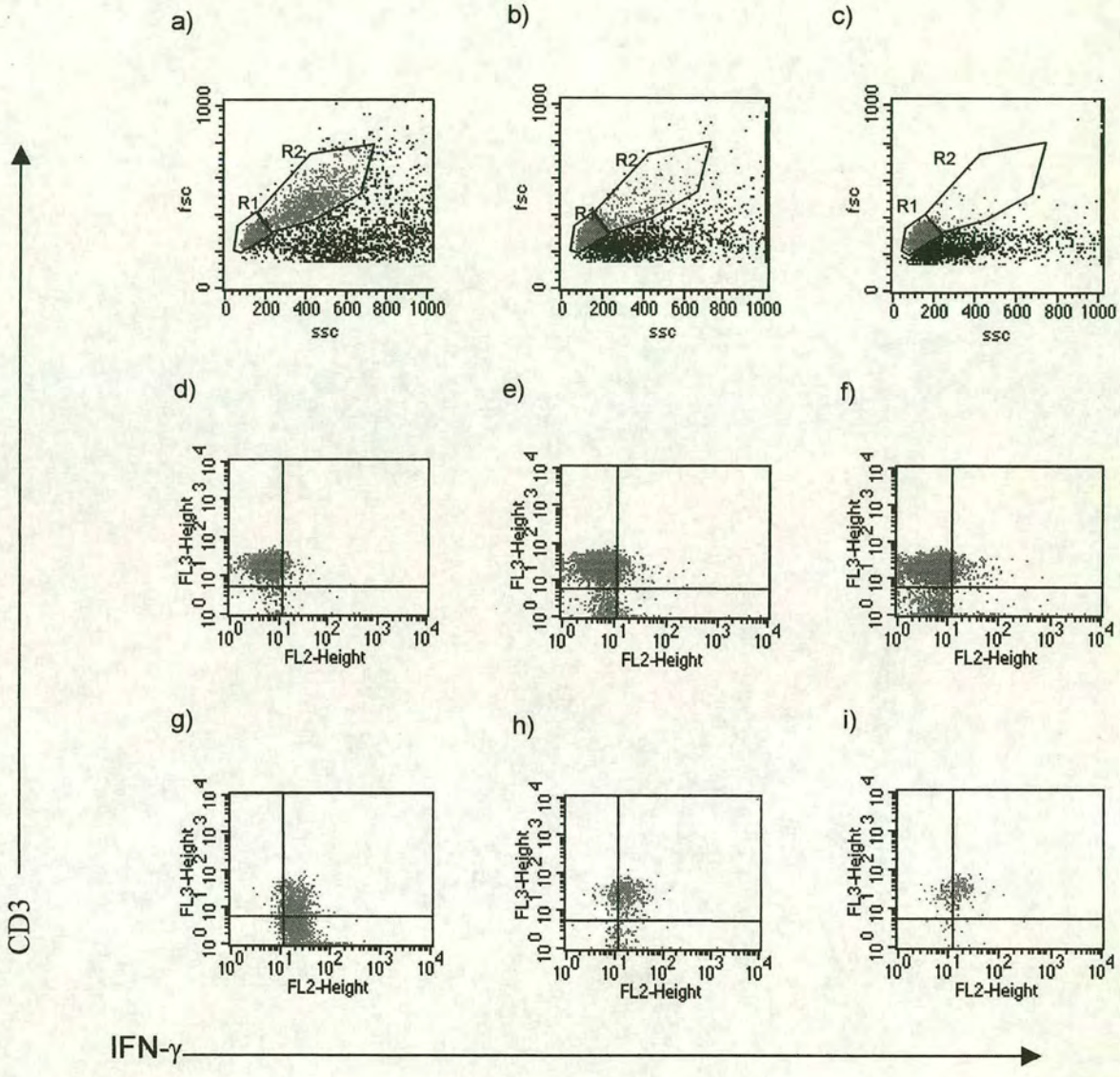

Figure 5